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*Hayathur Ben*

*July 27<sup>th</sup> 00*

PI - Signature

Date

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### A. Manuscripts

1. **Devi, G.R.**, Yang, D.Y., Rosenfeld, R.G. and Oh, Y. Differential effects of IGFBP-3 and its proteolytic fragments on ligand binding, IGFIR signaling and cell surface association. *Endocrinology, in press.*
2. Rosenfeld, R.G., Hwa, V., Wilson, L., Lopez-Bermejo, A., Buckway, C., Choi., **Devi, G.**, Ingermann, A., Graham, D., Minniti, G., Spagnoli, A., Oh, Y. (1999) The Insulin-like Growth Factor Binding Protein Superfamily: New Perspectives. *Pediatrics*.104(4 Pt 2):1018-1021.
3. Lopez-Bermejo, A., Buckway, C., **Devi, G.R.**, Hwa, V., Oh, Y., Plymate, S. and Rosenfeld, R.G. Characterization of IGFBP-related proteins in human prostate cells. *Endocrinology, in press.*

### B. Published Abstracts

1. **Devi, G.R.**, Sprenger, C., Graham, D.L., Plymate, S.R. and Rosenfeld, R.G.IGFBP-3 overexpression induces early apoptosis in malignant prostate cancer cells. 4<sup>th</sup> International IGFBP Symposia, Oct'00.
2. **Devi, G.R.**, Oh, Y. and Rosenfeld, R.G. Effect of IGFBP-3 on IGF- and IGF-analog-induced IGFIR signaling. (2000) Endocrine Society Proceedings.
3. **Devi, G.R.**, Yang, D.Y., Rosenfeld, R.G. and Oh, Y. Characterization of IGFs and insulin ligand binding to the amino and carboxyl-terminal IGFBP-3 proteolytic fragments' (1999) Endocrine Society Proceedings.

### C. Proof of new proposal submitted

## Annual Report (July 1999-June 2000)

### Introduction

Prostate cancer is the most commonly diagnosed cancer in males over the age of 50. Cancer growth involves loss of the normal growth regulatory machinery, which encompasses complex regulation of androgenic hormones and multiple growth factors, such as TGF- $\beta$  (transforming growth factor  $\beta$ ), EGF (epidermal growth factor), FGFs (fibroblast growth factors) and IGFs (insulin-like growth factors). IGFs (IGF-I and -II) are small peptides that are mitogenic for many cell types, including normal and malignant prostate cells. The presence of the full IGF-IGF receptors-IGF binding proteins (IGFBPs)-IGFBP-protease system within the prostate and the importance of the IGF-axis in the regulation of prostate growth has been well characterized in our laboratory. In the prostate, IGFBP-3, the predominant IGF carrier protein, has been shown to function as a modulator of IGF availability and bioactivity. In addition to these IGF-dependent actions, IGFBP 3 has direct anti-proliferative functions in the prostate, presumably through binding to specific cell surface receptors, and through mediating the anti-proliferative actions of other hormones. Further, in prostate cancer, IGFBP-3 undergoes proteolysis by prostate specific antigen (PSA), a serine protease and a critical prostate cancer marker. Therefore, limited proteolysis of IGFBP-3 appears to be a fundamental mechanism in regulating both the bioavailability of IGFs and the direct growth inhibitory function of IGFBP-3 on prostate cells. This study was initiated to understand the physiological importance of the intact IGFBP-3 and the IGFBP-3 proteolytic fragments generated by PSA, as modulators of prostate cell growth. **The central hypothesis is that proteolysis of IGFBP-3 modulates the biological action of IGFBP-3 on prostate cancer cell growth.** The specific aims of this study were 1.Characterization of the ligand binding and cellular functions of IGFBP-3 proteolytic fragments, 2. Characterization of the mechanism of action of IGFBP-3 and its proteolytic fragments. 3. Characterization of the effect of endogenous IGFBP-3 in prostate cells and in an *in vivo* mouse model of prostate cancer.

## I. Characterization of the ligand binding and cellular functions of IGFBP-3 proteolytic fragments

The insulin-like growth factors (IGF-I and -II) play an active role in cell proliferation and exist in association with distinct and specific IGF binding proteins designated as IGFBPs 1-6 (1) and possibly IGFBP-related proteins, IGFBP-rPs (2). IGFBP-3, the major IGFBP in adult serum, binds both IGFs with high affinity and specificity, and serves as a carrier of IGFs, prolonging their half lives, as well as modulating their proliferative and anabolic effects on target cells by regulating IGF bioavailability. Exogenous IGFBP-3 has also been demonstrated to significantly inhibit the growth of various cells, including Hs578T estrogen receptor-negative human breast cancer cells (3). Decreased cell growth was observed when human IGFBP-3 cDNA was transfected into mouse Balb/c fibroblast cells (4) and into fibroblast cells derived from mouse embryos homozygous for a targeted disruption of the type I IGF receptor gene (5). The mechanism of this inhibition appears to be both IGF-independent and IGF receptor-independent, and is mediated, presumably, through binding to specific IGFBP-3 receptors (6-8).

IGFBP-3 may be post-translationally modified by IGFBP-3 protease(s) present in biological fluids or culture media (plasmin, prostate-specific antigen, matrix metalloproteases) (9), and those whose activity has been demonstrated only *in vitro* like that of stromelysin 3, thrombin (10). Serum IGFBP proteases(s) have been detected in diabetes (11,12), renal (13), pregnancy (14), malignancy (15,16) and following traumatic conditions or invasive procedures, such as surgery. Cleavage sites in IGFBP-3 have been located at the beginning of the variable domain (residues 95-98), particularly residue 97 which is the cleavage site for PSA, plasmin, human serum and thrombin yields a fragment of approximately 16kDa or 20 kDa (glycosylated IGFBP-3) (10). However, the COOH-terminal fragments containing a highly basic heparin-binding domain, have only been detected *in vitro* by plasmin digestion of intact IGFBP-3 and these fragments seem to inhibit degradation of other binding proteins (17). It is recognized that IGFBP proteolysis also occurs in the normal state outside of the bloodstream (18,19) and that, in the cell environment, it is an essential mechanism in regulating the bioavailability of IGF. Both intact IGFBP-3 and IGFBP-3 proteolytic fragments have been shown to be capable of blocking the mitogenic effect of IGFs (20). Whether these actions primarily represent IGF-dependent or IGF-independent remains to be determined.

It has been hypothesized that the conserved NH<sub>2</sub>- and COOH-terminal sequences, as well as the appropriate ternary structure formed by disulfide bonds in the six classical IGFBPs, are all required for high affinity binding of IGFs. A recent study had indicated that a natural COOH-terminal fragment of human IGFBP-2 retained partial IGF-binding activity (23), and a COOH-terminal, 13kDa IGFBP-5 fragment isolated from hemofiltrate showed similar results (24). However, there is limited information on the binding characteristics of the IGFBP-3 COOH-terminal domain and the resultant biological effects of proteolytic fragments containing either the NH<sub>2</sub>- or COOH-terminal residues.

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In this study, we demonstrate the ability of COOH-terminal fragments of IGFBP-3 to bind IGFs. The (98-264)IGFBP-3 fragment and the (1-97)NH<sub>2</sub>-terminal fragment are both characterized, furthermore, by the ability to bind insulin with low affinity, but with higher affinity than is the case for intact IGFBP-3. Additionally, we have examined the effect of intact IGFBP-3 and the IGFBP-3 fragments on IGF-I-stimulated autophosphorylation of the IGFIR  $\beta$  subunit and their ability to associate with the cell surface.

Based on the ascertained and predicted recognition sites for known IGFBP-3 proteases, FLAG-epitope tagged intact IGFBP-3, NH<sub>2</sub>-terminal (1-97), intermediate fragment (88-148) and COOH-terminal fragments (98-264 and 184-264) were generated in a baculovirus and/or *E coli*. expression system and examined for their ability to bind IGF and insulin by western ligand blot and affinity cross-linking assays. The NH<sub>2</sub>- and COOH-terminal fragments bound both IGF and insulin specifically, albeit with significantly reduced affinity, for IGF but higher affinity for insulin, when compared to intact IGFBP-3. The effect of IGFBP-3 and the fragments on IGF-I receptor signaling pathways was studied by testing IGF-I-induced receptor autophosphorylation in IGFIR-overexpressing NIH-3T3 cells. IGFBP-3 showed a dose-dependent inhibition of autophosphorylation of the  $\beta$ -subunit of IGFIR. The (1-97)NH<sub>2</sub>-terminal fragment inhibited IGFIR autophosphorylation at high concentrations and this effect appears largely due to sequestration of IGF-I. In contrast, no inhibition of IGF-I-induced IGFIR autophosphorylation was detectable with the (98-264) and (184-264) COOH-terminal fragments, despite their ability to bind IGF. However, unlike the (1-97)NH<sub>2</sub>-terminal fragment, the COOH-terminal fragments of IGFBP-3 retained their ability to associate with the cell surface and this binding was competed by heparin, similar to intact IGFBP-3.

**Details of this study can be found in the attached manuscript.**

**Devi, G.R., Yang, D.Y., Rosenfeld, R.G. and Oh, Y.** Differential effects of IGFBP-3 and its proteolytic fragments on ligand binding, IGFIR signaling and cell surface association. *Endocrinology*, *in press*.

## **II. Characterization of the mechanism of action of IGFBP-3 and its proteolytic fragments.**

IGFBP-3 binds IGF-I and IGF-II with high affinity, at least an order of magnitude higher than the affinity of the IGFs for the IGFIR. It has been hypothesized that IGFBP-3 might inhibit IGF binding to the IGFIR via a mechanism independent of its ability to sequester IGFs. In the present study, we examined the effects of IGFBP-3 and its proteolytic fragments on the initial events of IGFIR signaling pathway. IGFBP-3 and the peptides were preincubated at 4°C and then added to NIH-3T3-IGFIR cells. Interestingly, IGFBP-3 inhibited IGF-I-, -II-, Des(1-3)IGF-I- and Long R3-IGF-I-induced IGFIR-P and IRS-I phosphorylation in a dose-dependent manner at similar concentration range (IC<sub>50</sub> range=5-7 nM). The (1-97)IGFBP-3 fragment was able to inhibit IGF-I-induced IGFIR-P but not Des-induced IGFIR-P. (1-97)IGFBP-3 fragment and not IGFBP-3 inhibited insulin-induced IGFIR-P. IGFIR-P stimulated by QAYL-IGF-II analog (very low affinity for IGFBP-3) was not inhibited by IGFBP-3. Preincubation of cells with IGFBP-3, followed by washing and IGF-I or IGF-analog treatment caused no inhibition of IGFIR-P, suggesting that IGFBP-3 requires ligand binding to suppress receptor activation. Monolayer

cross-linking with  $^{125}\text{I}$ -IGFBP-3 or unlabelled IGFBP-3 indicated that there is no direct interaction of IGFBP-3 with the receptor. This study clearly demonstrates that the effect on the initial step of IGFIR signaling by IGFBP-3 is purely due to its ability to sequester IGF and the IGF analogs in the extracellular milieu and not due to any interaction of IGFBP-3 with the IGFIR or a mechanism independent of its ability to bind IGFs. Further, this study reveals the differential binding ability of the (1-97)IGFBP-3 fragment for insulin.

**Devi, G.R.,** Graham, D.L., Oh, Y. and Rosenfeld, R.G. Effect of IGFBP-3 on IGF- and IGF-analog-induced IGFIR signaling (in preparation)

**\*An abstract of this work was published in the Endocrine Society Proceedings, 2000 and was an invited Oral presentation at the ENDO'00 meeting at Toronto, Canada (attached).**

### **III. Characterization of the effect of endogenous IGFBP-3 in prostate cells and in an *in vivo* mouse model of prostate cancer.**

Insulin-like growth factor binding protein (IGFBP-3) is secreted by prostate cell cultures. However, the IGFBP-3 levels are significantly reduced in malignant prostate epithelial cells (PEC) and in serum of patients with prostate cancer. Exogenous IGFBP-3 has been shown to block IGF action, inhibiting cell growth *in vitro*. The present study was carried out to evaluate the role of endogenous IGFBP-3 on prostate cell growth. M12 cells, a tumorigenic and metastatic PEC, was stably transfected with human IGFBP-3 cDNA (M12-BP-3) or with the vector pCDNA3 as control (M12-pCD). M12-BP-3 cells secreted IGFBP-3, whereas it was undetectable in the control cells.

Growth of M12-BP-3 cells was significantly slower and growth arrest occurred at a cell density that was 3 -fold lower than the control cells. There was a marked alteration in the M12-BP-3 cell morphology from the characteristic cuboidal shape associated with epithelial cells to elongated with increased cytoplasmic/nuclear ratios. Co-culture experiments in which the control M12 and M12-pCD cells were grown in conditioned media secreted by M12-BP-3 cells also showed altered morphology and increased number of detached cells within 12-24 h of treatment, which was reversed on removal of the media containing BP-3. A neuroendocrine marker, NSE, which is highly expressed in malignant prostate cells, was decreased in M12-BP-3 and in the control cells growing in BP-3 conditioned media. Altered mitochondrial membrane potential, an early apoptotic event, and an increased PARP cleavage in response to 6-hydroxyurea, was observed in M12-BP-3 cells. Flow cytometric analysis of the adherent and detached cells revealed that within 24 h, 20-30% of the M12-BP-3 cells were in pre-G1 peak, a growth characteristic of apoptotic cells, compared to 2% in the case of M12-pCD. These data indicate that endogenous IGFBP-3 causes significant delay in the growth of malignant PEC and enhances the sensitivity of these cells to apoptosis.



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Groups of 4-10 nude athymic male mice were injected s.c. with the M12-BP-3 or the control vector transfected cells. Mice were then monitored biweekly for tumor formation. Preliminary data reveals that the mice injected with either M12 cells or the vector transfected cells developed tumors 9/10 within 1-2 wks of injection. On the other hand, only 1/10 mice injected with M12-BP-3 cells developed a small tumor at 4 wks and the rest are tumor-free.

**An abstract of this work will be published in the Proceedings of the 4<sup>th</sup> International IGFBP Symposium, Terrigal. NSW, Australia. This work has also been selected for a presentation at the above symposia (*please see attached abstract*).**

**Devi, G.R., Sprenger, C., Plymate, S.R. and Rosenfeld, R.G. IGFBP-3 overexpression induces early apoptosis in malignant prostate cancer cells (*Manuscript in preparation*).**

## KEY SCIENTIFIC ACHEIVEMENTS

### \*HONORS

- 2000           Invited presentation and Travel Grant Award to attend The 4th International IGFBP'00 Symposium, Terrigal, NSW, Australia.
- 2000           Invited Speaker and Endocrine Society Travel Grant Award to present at Endo'00, Toronto, Canada.
- 1999-           Journal Reviewer  
                  J of Endocrinology and Endocrinology

**I was also involved in a collaborative project which looked at the function of recently identified members of the IGFBP superfamily, IGFBP-rP1 and IGFBP-rP2. This work has led to the following publications.**

Lopez-Bermejo, A., Buckway, C., **Devi, G.R.**, Hwa, V., Oh, Y., Plymate, S. and Rosenfeld, R.G. Characterization of IGFBP-related proteins in human prostate cells. *Endocrinology*, *in press*.

Lopez-Bermejo, A., **Devi, G.R.**, Buckway, C., Hwa, V., Oh, Y., Plymate, S. and Rosenfeld, R.G. Insulin-like growth factor binding protein-related protein 2: A new regulator of epithelial growth? (2000) *J Invest. Medicine*. 48(1):14A.

**PI: Gayathri R. Devi, Ph.D.**

## **REPORTABLE OUTCOMES**

### **\*PUBLICATIONS**

#### **A) Full-Length Papers**

1. **Devi, G.R.**, Yang, D.Y., Rosenfeld, R.G. and Oh, Y. Differential effects of IGFBP-3 and its proteolytic fragments on ligand binding, IGFIR signaling and cell surface association. *Endocrinology*, *in press*.

#### **B) Review Article**

Rosenfeld, R.G., Hwa, V., Wilson, L., Lopez-Bermejo, A., Buckway, C., Choi., **Devi, G.**, Ingermann, A., Graham, D., Minniti, G., Spagnoli, A., Oh, Y. (1999) The Insulin-like Growth Factor Binding Protein Superfamily: New Perspectives. *Pediatrics*.104(4 Pt 2):1018-1021.

#### **C) Manuscripts in preparation**

**Devi, G.R.**, Graham, D.L., Oh, Y. and Rosenfeld, R.G. Effect of IGFBP-3 on IGF- and IGF-analog-induced IGFIR signaling (in preparation).

**Devi, G.R.**, Sprenger, C., Graham, D.L., Plymate, S.R. and Rosenfeld, R.G. IGFBP-3 overexpression induces early apoptosis in malignant prostate cancer cells (in preparation).

**Devi, G.R.** and Rosenfeld, R.G. IGFBP-rP1 signaling by both IGF-dependent and IGF-independent mechanisms (in preparation).

**\* Invited Scientific Presentations**

- 2000 To present at the 4<sup>th</sup> International IGFBP Symposia, Oct'00, Terrigal, NSW, Australia. 'IGFBP-3 overexpression induces early apoptosis in malignant prostate cancer cells'.
- 2000 Oral presentation at the 82<sup>nd</sup> annual meeting of the Endocrine Society, Toronto, Canada. 'Effect of IGFBP-3 on IGF- and IGF-analog-induced IGFIR signaling'.
- 1999 Poster presentation at the 81st annual meeting of the Endocrine Society, San Diego, CA. 'Characterization of IGFs and Insulin Ligand binding to the Amino and Carboxyl-Terminal IGFBP-3 Proteolytic fragments'.

**\*Published Abstracts**

**Devi, G.R.,** Sprenger, C., Graham, D.L., Plymate, S.R. and Rosenfeld, R.G.IGFBP-3 overexpression induces early apoptosis in malignant prostate cancer cells. 4<sup>th</sup> International IGFBP Symposia, Oct'00.

**Devi, G.R.,** Oh, Y. and Rosenfeld, R.G. Effect of IGFBP-3 on IGF- and IGF-analog-induced IGFIR signaling. (2000) Endocrine Society Proceedings.

**Devi, G.R.,** Yang, D.Y., Rosenfeld, R.G. and Oh, Y. Characterization of IGFs and insulin ligand binding to the amino and carboxyl-terminal IGFBP-3 proteolytic fragments' (1999) Endocrine Society Proceedings.

**PI: Gayathri R. Devi, Ph.D.**

**\* Funding (pending)**

I have submitted a proposal to the Congressionally Directed Medical Research Program 2000 Prostate cancer Research Program in the **New Investigator Grant** category. Some of the work generated during my post-doctoral research was used as preliminary data (*please see attached document*).

**\* New Employment**

My current position as a Molecular Endocrinologist and Cancer Biologist at AVI BioPharma, a biopharmaceutical company was offered to me based on my post-doctoral research work in Dr. Ron Rosenfeld's laboratory.

July 2000-     Senior Scientist  
                 Molecular Endocrinologist and Cancer Biologist  
                 Department of Biology  
                 Gene Therapy and Cancer Division  
                 AVI BioPharma, Inc  
                 4575 SW Research way, Suite 200  
                 Corvallis, OR-97333

Responsible for initiating, directing and executing all preclinical scientific research and/or development strategies for treatment of prostate cancer. Serve as in-house and outside consultant and act as a spokesperson on corporate research and development and advise top management. Participate in development of patent applications and responsible for the management and professional development of the research group.

## CONCLUSION

The present study demonstrates that IGFBP-3 causes growth inhibition and induces early apoptosis in malignant prostate cancer cells and decreased tumor formation in male athymic nude mice. The mechanism of action of IGFBP-3 seems to lie in its ability to inhibit IGFIR activation, thereby modulating the mitogenic effects of IGF. This seems to be mediated primarily by binding and sequestration of IGFs by IGFBP-3 and not due to any direct interaction of IGFBP-3 with the IGFIR. Based on the ascertained and predicted recognition sites for known IGFBP-3 proteases like prostate specific antigen, metalloproteases, recombinant IGFBP-3 and its fragments were generated. In this study we clearly demonstrate the ability of the IGFBP-3 amino-terminal fragment to bind IGF and insulin and to inhibit IGFIR and insulin receptor autophosphorylation, revealing that this 16 kDa fragment may be capable of both IGF-I-dependent and IGF-independent roles in modulating cell growth. However, the carboxyl-terminal fragments, which also have the ability to bind both IGF-I and insulin *in vitro*, fail to prevent binding of either IGF-I or insulin to their respective receptors, due to the tendency of these fragments for cell surface association via the heparin binding domain. In summary, these data suggest that IGFBP-3 has a suppressive effect on prostate cancer development and various forms of IGFBP-3 fragments resulting from proteolysis by IGFBP-3-specific proteases may have different effects on the IGF-IGFIR axis, as well as potential IGF-independent actions.

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3. **Oh Y, Muller HL, Lamson G and Rosenfeld RG** 1993 Insulin-like growth factor (IGF)-independent action of IGF-binding protein-3 in Hs578T human breast cancer cells. *J. Biol. Chem.* 268:14964-14971
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20. **Lalou C, Lassarre C and Binoux M** 1996 A proteolytic fragment of insulin-like growth factor (IGF) binding protein-3 that fails to bind IGFs inhibits the mitogenic effects of IGF-I and insulin. *Endocrinology* 137:3206-3212
21. **Yamanaka Y, Wilson EM, Rosenfeld RG and Oh Y** 1997 Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. *J. Biol. Chem.* 272:30729-30734
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23. **Ho PJ and Baxter RC** 1997 Characterization of truncated insulin-like growth factor-binding protein-2 in human milk. *Endocrinol.* 138:3811-3818

# APPENDICES

## A. Manuscripts

1. **Devi, G.R.**, Yang, D.Y., Rosenfeld, R.G. and Oh, Y. Differential effects of IGFBP-3 and its proteolytic fragments on ligand binding, IGFIR signaling and cell surface association. *Endocrinology*, *in press*.
2. Rosenfeld, R.G., Hwa, V., Wilson, L., Lopez-Bermejo, A., Buckway, C., Choi., **Devi, G.**, Ingermann, A., Graham, D., Minniti, G., Spagnoli, A., Oh, Y. (1999) The Insulin-like Growth Factor Binding Protein Superfamily: New Perspectives. *Pediatrics*.104(4 Pt 2):1018-1021.
3. Lopez-Bermejo, A., Buckway, C., **Devi, G.R.**, Hwa, V., Oh, Y., Plymate, S. and Rosenfeld, R.G. Characterization of IGFBP-related proteins in human prostate cells. *Endocrinology*, *in press*.

## B. Published Abstracts

1. **Devi, G.R.**, Sprenger, C., Graham, D.L., Plymate, S.R. and Rosenfeld, R.G. IGFBP-3 overexpression induces early apoptosis in malignant prostate cancer cells. 4<sup>th</sup> International IGFBP Symposia, Oct'00.
2. **Devi, G.R.**, Oh, Y. and Rosenfeld, R.G. Effect of IGFBP-3 on IGF- and IGF-analog-induced IGFIR signaling. (2000) Endocrine Society Proceedings.
3. **Devi, G.R.**, Yang, D.Y., Rosenfeld, R.G. and Oh, Y. Characterization of IGFs and insulin ligand binding to the amino and carboxyl-terminal IGFBP-3 proteolytic fragments' (1999) Endocrine Society Proceedings.

## C. Proof of new proposal submitted for funding

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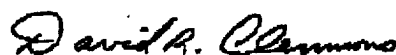
**Differential effects of IGFBP-3 and its proteolytic fragments on ligand binding, cell surface association and IGFIR signaling**

Dear Dr. Devi:

We are pleased to inform you that your revised manuscript has been accepted for publication in the November 2000 issue of Endocrinology. We congratulate you and look forward to future submissions.

We would also like to take this opportunity to remind you of the publication charges. Page charges of \$85.00 per page and, if applicable, additional charges of \$750.00 per color plate will be billed to you by Cadmus Journal Services at the time of receipt of your page proofs (approximately one month before the publication date). If you do not receive page proofs in about 12 weeks, you may contact Trisha Gage at Cadmus Journal Services, Phone (410) 691-6433, or Fax (410) 684-2791.

Sincerely,



David R. Clemmons  
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**Differential Effects of IGFBP-3 and its Proteolytic Fragments on Ligand Binding, Cell  
Surface Association and IGFIR Signaling**

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**Running Title:** The Effect of IGFBP-3 Fragments on IGFIR Signaling

## ABSTRACT

Insulin-like growth factor binding protein-3 (IGFBP-3), the predominant IGF carrier protein in circulation, is post-translationally modified *in vivo* by IGFBP-3 protease(s) into a number of fragments. Based on the ascertained and predicted recognition sites for known IGFBP-3 proteases, FLAG-epitope tagged intact IGFBP-3, NH<sub>2</sub>-terminal (1-97), intermediate fragment (88-148) and COOH-terminal fragments (98-264 and 184-264) were generated in a baculovirus and/or *E. coli*. expression system and examined for their ability to bind IGF and insulin by western ligand blot and affinity cross-linking assays. The NH<sub>2</sub>- and COOH-terminal fragments bound both IGF and insulin specifically, albeit with significantly reduced affinity, for IGF but higher affinity for insulin, when compared to intact IGFBP-3. The effect of IGFBP-3 and the fragments on IGF-I receptor signaling pathways was studied by testing IGF-I-induced receptor autophosphorylation in IGFBP-3 overexpressing NIH-3T3 cells. IGFBP-3 showed a dose-dependent inhibition of autophosphorylation of the  $\beta$ -subunit of IGFBP-3. The (1-97)NH<sub>2</sub>-terminal fragment inhibited IGFBP-3 autophosphorylation at high concentrations and this effect appears largely due to sequestration of IGF-I. In contrast, no inhibition of IGF-I-induced IGFBP-3 autophosphorylation was detectable with the (98-264) and (184-264) COOH-terminal fragments, despite their ability to bind IGF. However, unlike the (1-97)NH<sub>2</sub>-terminal fragment, the COOH-terminal fragments of IGFBP-3 retained their ability to associate with the cell surface and this binding was competed by heparin, similar to intact IGFBP-3.

## INTRODUCTION

The insulin-like growth factors (IGF-I and -II) play an active role in cell proliferation and exist in association with distinct and specific IGF binding proteins designated as IGFBPs 1-6 (1) and possibly IGFBP-related proteins, IGFBP-rPs (2). IGFBP-3, the major IGFBP in adult serum, binds both IGFs with high affinity and specificity, and serves as a carrier of IGFs, prolonging their half lives, as well as modulating their proliferative and anabolic effects on target cells by regulating IGF bioavailability. Exogenous IGFBP-3 has also been demonstrated to significantly inhibit the growth of various cells, including Hs578T estrogen receptor-negative human breast cancer cells (3). Decreased cell growth was observed when human IGFBP-3 cDNA was transfected into mouse Balb/c fibroblast cells (4) and into fibroblast cells derived from mouse embryos homozygous for a targeted disruption of the type I IGF receptor gene (5). The mechanism of this inhibition appears to be both IGF-independent and IGF receptor-independent, and is mediated, presumably, through binding to specific IGFBP-3 receptors (6-8).

IGFBP-3 may be post-translationally modified by IGFBP-3 protease(s) present in biological fluids or culture media (plasmin, prostate-specific antigen, matrix metalloproteases) (9), and those whose activity has been demonstrated only *in vitro* like that of stromelysin 3, thrombin (10). Serum IGFBP proteases(s) have been detected in diabetes (11,12), renal (13), pregnancy (14), malignancy (15,16) and following traumatic conditions or invasive procedures, such as surgery. Cleavage sites in IGFBP-3 have been located at the beginning of the variable domain (residues 95-98), particularly residue 97 which is the cleavage site for PSA, plasmin, human serum and thrombin yields a fragment of approximately 16kDa or 20 kDa (glycosylated IGFBP-3) (10). However, the COOH-terminal fragments containing a highly basic heparin-binding domain, have only been detected *in vitro* by plasmin digestion of intact IGFBP-3 and these fragments seem to inhibit degradation of other binding proteins (17). It is recognized that

IGFBP proteolysis also occurs in the normal state outside of the bloodstream (18,19) and that, in the cell environment, it is an essential mechanism in regulating the bioavailability of IGF. Both intact IGFBP-3 and IGFBP-3 proteolytic fragments have been shown to be capable of blocking the mitogenic effect of IGFs (20). Whether these actions primarily represent IGF-dependent or IGF-independent remains to be determined.

Our laboratories have demonstrated that the NH<sub>2</sub>-terminal recombinant fragments of IGFBP-3, (1-87) and (1-97) retain the ability to bind IGF, albeit with substantially reduced affinity. Additionally, these fragments specifically bind insulin and modulate insulin binding to its receptor (21,22). Based on these studies, it has been hypothesized that the conserved NH<sub>2</sub>- and COOH-terminal sequences, as well as the appropriate ternary structure formed by disulfide bonds in the six classical IGFBPs, are all required for high affinity binding of IGFs. A recent study had indicated that a natural COOH-terminal fragment of human IGFBP-2 retained partial IGF-binding activity (23), and a COOH-terminal, 13kDa IGFBP-5 fragment isolated from hemofiltrate showed similar results (24). However, there is limited information on the binding characteristics of the IGFBP-3 COOH-terminal domain and the resultant biological effects of proteolytic fragments containing either the NH<sub>2</sub>- or COOH-terminal residues.

In this study, we demonstrate the ability of COOH-terminal fragments of IGFBP-3 to bind IGFs. The (98-264)IGFBP-3 fragment and the (1-97)NH<sub>2</sub>-terminal fragment are both characterized, furthermore, by the ability to bind insulin with low affinity, but with higher affinity than is the case for intact IGFBP-3. Additionally, we have examined the effect of intact IGFBP-3 and the IGFBP-3 fragments on IGF-I-stimulated autophosphorylation of the IGFIR  $\beta$  subunit and their ability to associate with the cell surface.

## MATERIALS and METHODS

**Antibodies and Reagents.** IGF-I and IGF-II were purchased from Austral Biologicals (Santa Clara, CA).  $^{125}\text{I}$ -IGF-I (specific activities between 50-70  $\mu\text{Ci}/\mu\text{g}$  by a modification of chloramine-T technique) and IGFBP-3 monoclonal antibody were kindly provided by Diagnostic System Laboratories, TX. IGFBP-3<sup>E.coli</sup> was obtained from Celtrix (Santa Clara, CA).  $^{125}\text{I}$ -(A14)-monoiodinated insulin was purchased from Amersham Corp. Bovine insulin was purchased from Sigma. Anti-phosphotyrosine monoclonal antibody (4G10) was a generous gift from Dr. B. J. Druker (Dept. of Hematology and Medical Oncology, Oregon Health Sciences University). Reagents used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Bio-Rad Laboratories (Richmond, CA).

**Cell Culture.** NIH-3T3 cells overexpressing the human IGF-I receptor were kindly gifted from Dr. C.T. Roberts, Jr (Dept. of Pediatrics, Oregon Health Sciences University) and grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum plus 500  $\mu\text{g}/\text{ml}$  geneticin at 37°C with 5% CO<sub>2</sub>.

### *Generation and Purification of Recombinant IGFBP-3 Proteolytic Fragments.*

(1-97)IGFBP-3 and (88-148)IGFBP-3 FLAG-epitope tagged fragments were generated and purified in baculovirus and tested to be 99% pure as described earlier (25,26). The cDNAs for the COOH-terminal fragment- (98-264) and (184-264) were generated by PCR amplification from the human IGFBP-3 cDNA and a FLAG epitope sequence (DYKDDDDK) and a stop codon was added immediately following amino acid 264. The signal peptide sequences of IGFBP-3 cDNA were ligated to NH<sub>2</sub>-termini of IGFBP-3 fragments. After sequencing, the 98-264 amplicon was then subcloned into the baculovirus expression vector pFASTBAC1 (Life



Technologies, Inc.) and transformed into DH10Bac *E. coli* cells. The amplified DNA was transfected into Sf9 insect cells (ATCC) and large scale protein purification was begun by infecting the P2 virus into  $10^8$  HI-5 insect cells (Invitrogen) at a multiplicity of infection of 3 at 27°C for 3 days. The media from the infected cells were collected and concentrated, and the resultants were bound to an anti-M2 antibody column overnight at 4°C and the FLAG tagged (98-264) protein was then eluted by using FLAG peptide (0.5 µg/ml) as described earlier (27). The purified protein was subjected to SDS-PAGE in a 15% gel, and stained with Coomassie blue. Further, the fragment was also identified by immunoblotting with the M2 anti-FLAG antibody (Eastman Kodak, New Haven, CT) and anti-IGFBP-3 monoclonal antibody (DSL, Texas). Eluted fractions from an anti-M2 antibody column were pooled, concentrated, and quantitated by comparison with known amounts of bovine serum albumin and IGFBP-3<sup>E.coli</sup> after silver staining.

The (184-264)IGFBP-3 amplicon after sequencing, was subcloned in the C-terminal end of glutathione S-transferase (GST) in the plasmid pGEX4T and transformed into *E. coli* cells. The culture was grown overnight in LB-ampicillin and induced with 2 mM IPTG and the cell lysates of the (184-264) GST fusion protein were prepared. The lysates were incubated with GST sepharose beads for 1 h at RT and then washed. Purity and concentration of the fragments were determined by comparison with known amounts of bovine serum albumin standards after silver staining. Further, the purified protein was subjected to SDS-PAGE in a 15% gel, and stained with Coomassie Blue and also transferred to nitrocellulose and identified by immunoblotting with M2 anti-FLAG antibody (1:3000 dilution).

**Affinity Cross-Linking.** Intact IGFBP-3 or the NH<sub>2</sub>- and COOH-terminal fragments were incubated with <sup>125</sup>I-IGF-I or <sup>125</sup>I-insulin (50,000 cpm), in the presence or absence of unlabeled ligand, in a 100 µl volume for 16 h at 4°C and then cross-linked with 0.5 mM DSS for 15 min at

4°C. The samples were then subjected to SDS-PAGE (12% or 15% gels) under reducing conditions, and autoradiography on Biomax MS film. Bands were quantified by densitometry (Bio-Rad).

**Western Ligand Blot Analysis.** Ligand blotting was performed as described by Hossenlopp et al. (28), with minor modifications. Briefly, samples of intact IGFBP-3, (1-97)IGFBP-3 and (98-264)IGFBP-3 fragments at the concentrations indicated in the figure legends were subjected to SDS-PAGE (12% or 15% gel) under reducing or non-reducing conditions, electroblotted onto nitrocellulose filters, incubated with  $1.5 \times 10^6$  cpm of  $^{125}\text{I}$ -insulin or a mixture of  $^{125}\text{I}$ -IGF-I and  $^{125}\text{I}$ -IGF-II, washed, dried and exposed to film (Biomax, Eastman Kodak).

**Monolayer  $^{125}\text{I}$ -IGF-I Affinity Cross-linking.**  $^{125}\text{I}$ -IGF-I (100,000 cpm) was preincubated in a microfuge tube for 2 h at 4°C, in the presence or absence of cold IGF-I, intact IGFBP-3 (30 nM), (98-264)IGFBP-3 (250 nM), (184-264)IGFBP-3 (250 nM) or (1-97)IGFBP-3 (250 nM), in binding buffer- 50 mM Hepes, 150 mM NaCl, 0.5% bovine serum albumin. Confluent NIH-3T3 cells stably transfected with the human IGF-I receptor cDNA (NIH-3T3-IGFIR cells) were incubated in serum free medium overnight. The cells were washed once with phosphate buffered saline (PBS). The cells were incubated with the  $^{125}\text{I}$ -IGF-I/IGFBP-3 combinations in triplicate wells for 3 h at 15 °C. The cells were then washed with PBS and crosslinked with DSS for 15 min at 4 °C, and the reaction was quenched with 100 mM Tris/HCl. The cells were solubilized with sample buffer. The covalent ligand-receptor ( $^{125}\text{I}$ -IGF-I-IGFIR) complex in the lysates was resolved on a non-reducing 6% SDS-PAGE followed by autoradiography. Another set of the same samples of cell lysates were run on a 15% SDS-PAGE under reducing conditions and immunoblotted with M2 anti-FLAG antibody or anti-IGFBP-3 monoclonal antibody and the cell associated bands were detected with enhanced chemiluminescence (Amersham).

***Determination of Cell-Surface Association of Intact IGFBP-3 and the Fragments.*** Confluent monolayers of NIH-3T3-IGFIR cells were incubated in serum free medium overnight. Intact IGFBP-3 (30 nM), fragments; 98-264 (250 nM), 184-264 (250 nM) or 1-97 (250 nM) in the presence or absence of 100 µg/ml heparin (Sigma) in binding buffer was added to the cells. In a similar experiment, cells were treated with heparin (100 µg/ml) for 1 h before addition of the peptides as listed above. The treatments were carried out at 15 °C for 3 h. The cells were washed with PBS and cross-linked with DSS, as described above. The solubilized cell lysates were then run on a 15% SDS-PAGE and immunoblotted with anti-IGFBP-3 monoclonal antibody and detected with enhanced chemiluminescence.

***IGF-I-induced IGFIR Autophosphorylation Assay.*** Confluent monolayers of serum starved NIH-3T3-IGFIR cells were exposed for 5 min to 7 nM IGF-I, which had been preincubated with/without intact IGFBP-3, (1-97) IGFBP-3 or (98-264)IGFBP-3 for 2 h at 4 °C. The reaction was quenched by solubilization buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 12 units/ml aprotinin, phenylmethylsulfonyl flouride, and 1 mM Na<sub>3</sub>VO<sub>4</sub>). Solubilized proteins (25 µl of the cell lysates) were separated by SDS-PAGE (7.5%) under reducing conditions and visualized by immunoblot analysis. For immunoblot analysis, the filters were blocked in Tris-buffered saline (TBS) with 2% gelatin for 1 h at room temperature and then incubated with anti-phosphotyrosine monoclonal antibody (1.5 µg/ml) diluted by TBS + 0.1% Triton X-100 (TBST) for 1 h at room temperature. The filters were then rinsed in 1XTBST and incubated in a 1:5000 dilution of goat anti-mouse IgG-conjugated horseradish peroxidase (Amersham Corp.) for 1 h at room temperature. Immunoreactive proteins were visualized using enhanced chemiluminiscence detection system.

## RESULTS

### *Expression of the IGFBP-3 recombinant Fragments*

Based on the ascertained and predicted PSA recognition sites in IGFBP-3, and the recognition sites for other known IGFBP-3 proteases, such as metalloproteases and plasmin, both intact IGFBP-3 and four different recombinant fragments were generated in a baculovirus and/or *E. coli* expression system. Each peptide was coupled with a FLAG-epitope tag at the carboxy-terminus, as shown in Fig. 1A. The purified proteins were immunoblotted with anti-FLAG M2 or anti-IGFBP-3 monoclonal antibody for estimation of their molecular weights. Intact IGFBP-3 and all the fragments were detectable by anti-FLAG M2 antibody under reducing (Fig. 1B) and non-reducing conditions. Dimerized forms of the proteins were identified in anti-FLAG M2 immunoblots run under non-reducing conditions (data not shown). Small discrepancies between the  $M_r$  for intact IGFBP-3, (1-97)IGFBP-3, (98-264)IGFBP-3 and (88-148)IGFBP-3 proteins seen on the immunoblots relative to the predicted  $M_r$ , which is purely based on amino acid composition of the proteins, may have arisen due to N-linked glycosylation. There are three potential N-glycosylation sites (Fig. 1A), Asn<sup>89</sup>, Asn<sup>109</sup> and Asn<sup>172</sup> in IGFBP-3 (29). The anti-IGFBP-3 monoclonal antibody detected intact IGFBP-3 and (98-264)IGFBP-3 under both non-reducing (Fig. 1B) and reducing conditions (data not shown). The fragment 1-97 was detectable only under non-reducing conditions. The 184-264 and 88-148 fragments were not detected effectively with this antibody.

### *Analysis of IGF binding to the IGFBP-3 Proteolytic Fragments*

To determine whether the regions encompassed by the IGFBP-3 fragments contained a functional IGF-I binding/cross-linking site, the proteins were incubated with <sup>125</sup>I-IGF-I and then affinity cross-linked with DSS and analyzed by SDS-PAGE. The data in Fig. 2A demonstrate

that  $^{125}\text{I}$ -IGF-I can be cross-linked to the (1-97)IGFBP-3 and (98-264)IGFBP-3 fragments in a dose-dependent manner. Significant IGF cross-linking was observed at 50 nM concentrations of (1-97)IGFBP-3 which was completely saturated by 100 nM concentrations. In the case of (98-264)IGFBP-3 a dose-dependent increase in IGF binding with increasing protein concentrations with saturation of binding occurring by 500 nM concentration range. The expected sizes of the individual proteolytic fragment coupled to 7 kDa  $^{125}\text{I}$ -IGF-I were detected, shown as 25 and 41 kDa bands, respectively. A faint band at 39 kDa is potentially a dimerized form of 1-97 fragment cross-linked to IGF-I.

For estimation of the affinity of IGF-I binding, the proteolytic fragments were affinity cross-linked with  $^{125}\text{I}$ -IGF-I in the presence of increasing amounts of unlabeled IGF-I or insulin ranging between 0.15 - 4.5  $\mu\text{M}$ . A dose-dependent displacement of  $^{125}\text{I}$ -IGF-I labeling of the IGFBP-3 fragments 1-97 and 98-264 was observed with increasing concentrations of unlabeled IGF-I (Fig. 2B). Quantitative analysis of the radioactive bands in these gels was done by densitometric analysis and the data were plotted as a function of IGF-I or insulin concentration to construct  $^{125}\text{I}$ -IGF-I displacement curves (Fig. 2C). Calculation of the unlabeled IGF-I concentrations required to achieve 50% displacement of binding ( $\text{IC}_{50}$ ) indicated that (1-97)IGFBP-3 fragment had an  $\text{IC}_{50}$  value of 0.2-0.3  $\mu\text{M}$ . The (98-264)IGFBP-3 fragment binding to  $^{125}\text{I}$ -IGF-I was completely displaced by 0.15  $\mu\text{M}$  unlabeled IGF-I (Fig. 2B), and, using lower concentrations of unlabeled IGF-I in similar experiments, revealed the  $\text{IC}_{50}$  to be in the range of 0.1-0.15  $\mu\text{M}$  (Fig. 2C). In addition, insulin was also able to compete for  $^{125}\text{I}$ -IGF-I binding to the fragments, especially to (1-97)IGFBP-3. The  $\text{IC}_{50}$  values calculated for unlabeled insulin to compete for  $^{125}\text{I}$ -IGF-I binding revealed that the 1-97 fragment had an  $\text{IC}_{50}$  value of about 0.3  $\mu\text{M}$  insulin, which was at least 3 fold lower than that of the 98-264 fragment ( $\text{IC}_{50}$ =0.8-1  $\mu\text{M}$ ), as shown in Figure 2C. In summary, the (1-97)IGFBP-3 showed slightly lower binding affinity for

IGF-I relative to (98-264)IGFBP-3 and  $^{125}\text{I}$ -IGF-I binding to the fragments was equipotently displaced by insulin.

Further, the ability of the fragments to bind IGF-I and IGF-II was assessed by western ligand blotting. The data in figure 3 demonstrate that both the 1-97 and 98-264 fragments were able to bind to a mixture of IGF-I and IGF-II at concentrations as low as 5-10 pmoles. It is to be noted, however, that the intensity of the major radiolabeled bands was much lower than that observed with intact IGFBP-3 at similar concentrations. The 20 kDa faint band observed in the case of (1-97)IGFBP-3 is probably a different glycosylation product of the 16 kDa fragment.

#### *Analysis of Insulin Binding to IGFBP-3 Fragments*

The observations that insulin could compete for  $^{125}\text{I}$ -IGF-I binding to the fragments led us to assess their insulin binding activity. Both (1-97)IGFBP-3 and (98-264)IGFBP-3 showed a strong  $^{125}\text{I}$ -insulin cross-linking band in comparison to that observed with IGFBP-3 at similar concentrations (Fig. 4a). Unlabeled insulin was able to dose-dependently inhibit  $^{125}\text{I}$ -insulin binding to both the  $\text{NH}_2$  and  $\text{COOH}$ -terminal fragments (Fig. 4B), although even higher concentrations of unlabeled insulin could not completely displace  $^{125}\text{I}$ -insulin binding to the (98-264)IGFBP-3, suggesting a slow dissociation rate. Calculation of the unlabeled insulin concentrations required to achieve 50% displacement of binding ( $\text{IC}_{50}$ ) indicated that the (1-97)IGFBP-3 had an  $\text{IC}_{50}$  value ranging between 0.3-0.4  $\mu\text{M}$ , whereas nearly 1  $\mu\text{M}$  unlabeled insulin was required to cause 50% displacement of  $^{125}\text{I}$ -insulin binding to the (98-264) fragment (Fig. 4c). IGF-I was also able to compete for the  $^{125}\text{I}$ -insulin binding to the fragments, although higher concentrations of IGF-I was required in the case of (98-264)IGFBP-3. In summary, the (1-97)IGFBP-3 showed significantly high affinity for insulin relative to the (98-264)IGFBP-3 fragment.

Further, western blot analysis with iodinated insulin showed that both 1-97 and the 98-264 fragment bound insulin. The IGFBP-3 intermediate fragment (88-148), which lacks both the NH<sub>2</sub>- and COOH-terminal domains, showed no binding to either IGF-I, IGF-II or insulin in western ligand blot (data not shown).

#### ***IGFBP-3 and 1-97 Fragment Inhibit IGF-I interaction with the IGFIR***

To determine, whether the ability of intact IGFBP-3 and the amino- and carboxy-terminal fragments to bind IGFs *in vitro* lead to sequestration of IGFs *in vivo*, <sup>125</sup>I-IGF-I monolayer affinity cross-linking assay was done in the NIH-3T cells overexpressing the IGFIR (NIH-3T3-IGFIR). The data in Figure 5A shows that <sup>125</sup>I-IGF-I specifically crosslinks with the IGFIR shown as 230 kDa band under non-reducing conditions. <sup>125</sup>I-IGF-I binding to IGFIR was completely displaced by 100 nM unlabeled IGF-I. Further, the IGF-I-IGFIR complex formation was completely inhibited by preincubation of the iodinated IGF-I with unlabeled IGFBP-3 (30 nM) and about 90% inhibited by preincubating with 250 nM concentration of (1-97) NH<sub>2</sub>-terminal fragment. The cross-linked band was not inhibited, however, by preincubation of the <sup>125</sup>I-IGF-I with 250 nM of (98-264)IGFBP-3 or the (184-264)IGFBP-3 fragment.

The same set of samples were resolved on an immunoblot and probed with M2 anti-FLAG antibody. Results in Figure 5B show that the carboxy-terminal fragments-(98-264), (184-264) and intact IGFBP-3 molecules associated with the cell surface in the presence of IGF-I. (1-97)IGFBP-3, however, showed no cell-associated band.

#### ***The Carboxy-Terminal Fragments have the Ability to Associate to the Cell Surface***

Since, compared to the NH<sub>2</sub>-terminal fragment, the (98-264) COOH-terminal IGFBP-3 fragment failed to inhibit binding of IGF-I to the IGFIR, we wanted to study the ability of the fragments to associate with the cell surface in the absence of IGF-I. Monolayer cross-linking was carried out with the FLAG-epitope tagged intact IGFBP-3, (1-97) or (98-264) fragments in NIH-3T3-IGFIR cells, and the cell-associated proteins were detected by immunoblotting the cell lysates with anti-IGFBP-3 monoclonal antibody. The (98-264) carboxy-terminal fragment and intact IGFBP-3 molecules associated with the cell surface. (1-97)IGFBP-3, however, showed no cell-associated band (Fig. 6; lanes 2, 5). Further, there was no detectable shift in molecular weights of the cross-linked proteins when compared with control (Fig. 1B) non-cross-linked protein preparations.

In order to test whether the ability of intact IGFBP-3 and the carboxyl-terminal fragment to bind to the cell surface was via the heparin binding domains, cells were preincubated with heparin (100 µg/ml) and then treated with the peptides; alternatively the peptides were preincubated with heparin and then added to the cells, followed by monolayer cross-linking in both cases (Fig. 6). Similar results were observed in both types of experiments, i.e., heparin blocked the cell surface association of intact IGFBP-3 (Fig. 6, lanes 3, 4) and the (98-264)IGFBP-3 fragment (Fig. 6, lanes 6,7).

### *Inhibition of IGFIR Signaling*

Since intact IGFBP-3 and its fragments have the ability to bind IGFs and thereby impede its interaction with the IGFIR, we analyzed the potential biological manifestation of this interaction on IGFIR signaling. This was carried out by testing the effect of IGFBP-3 and its fragments on IGF-I-induced IGFIR autophosphorylation in NIH-3T3-IGFIR cells. Control



experiments with IGF-I revealed that 5 min. treatments with 7-14 nM of the peptide showed maximal intensity autophosphorylation of the 95 kDa band of the  $\beta$  subunit of IGFIR in anti-phosphotyrosine immunoblots (Fig. 7A).

IGFBP-3 inhibited IGF-I-stimulated autophosphorylation of the IGFIR  $\beta$  subunit in a dose-dependent manner (Fig. 7B). Quantification of the inhibition of the phosphorylated subunit of IGFIR was carried out by densitometrically analyzing the specific 95 kDa band and the 116 kDa non-specific band in each gel. The ratio of the two band intensities was used to normalize and calculate the percentage of maximal IGF-I-stimulated IGFIR autophosphorylation detected in the presence of IGFBP-3 and the IGFBP-3 fragments (Fig. 7C). IGFBP-3 caused 50% inhibition of the IGF-I-induced autophosphorylation at 5-7 nM concentration range and by 15-20 nM IGFBP-3 concentrations, complete inhibition of IGFIR autophosphorylation was observed. In contrast, the (1-97)IGFBP-3 fragment inhibited receptor autophosphorylation only at higher concentrations (50-70% inhibition at 100-250 nM concentrations). The (98-264)IGFBP-3 and (184-264)IGFBP-3 fragments, however, did not show any significant inhibition of IGF-I-induced IGFIR autophosphorylation, even at 250 nM concentrations (Fig. 7B and 7C), although these fragments were able to bind IGF-I in binding assays.

## DISCUSSION

We report herein that IGFBP-3 fragments are capable of binding IGF-I and IGF-II, although with lower affinity than that seen with intact IGFBP-3. Further, the fragments have the ability to bind insulin with higher affinity than observed with intact IGFBP-3. The principal conclusion is that the high affinity binding of IGFs by IGFBP-3 requires proper tertiary configuration of the NH<sub>2</sub>- and COOH-terminal domains. This observation is further supported by the recent concept of an IGFBP superfamily (30,31). Over the course of evolution, the classical IGFBPs, which have well-conserved NH<sub>2</sub>- and COOH-terminal domains, evolved into high-affinity IGF-binders (1). In contrast, the IGFBP-rPs (low-affinity IGF binding proteins) only share the conserved NH<sub>2</sub>-terminal domain (32). This structural difference, combined with the present data, strongly implicate the importance of the IGFBP COOH-terminal domain in conferring high-affinity IGF binding.

The concept that interaction between NH<sub>2</sub>- and COOH-terminal domains is essential for high-affinity IGF binding was initially conceived based on observations that proteolysis of IGFBPs in biological fluids results in fragments which have diminished or no binding affinities for IGFs (33). The *in vitro* generation of recombinant fragments or fragments isolated by limited proteolysis supports the *in vivo* data. A 16 kDa fragment corresponding to the NH<sub>2</sub>-terminus and a small portion of intermediate region, generated by proteolytically modifying IGFBP-4, specifically cross-linked to both IGF-I and II, although with a 20-fold lower affinity than intact IGFBP-4 (34,35). Similarly, a carboxy-truncated 23 kDa IGFBP-5 fragment from osteoblast-like cells demonstrated decreased IGF binding affinity (36,37). Deletion mutagenesis of the carboxy-terminal domains of IGFBP-1 and IGFBP-4 have resulted in a decrease in IGF affinity, thereby demonstrating the importance of the highly conserved Cys-Try-Cys-Val motif in the carboxy-

terminal region (38,39). The present study is the first to clearly demonstrate the ability of the 28 kDa, (98-264)IGFBP-3 intermediate+COOH-terminal proteolytic fragment to bind both IGFs and insulin in two different procedures, affinity cross-linking and western ligand blot. Interestingly, the binding of the (98-264)IGFBP-3 fragment to  $^{125}\text{I}$ -IGF-I or  $^{125}\text{I}$ -insulin were competitively displaced by both IGF-I and insulin, though with different affinities, suggesting that the insulin and IGF binding site are probably not identical, but overlap or reside closely on the IGFBP-3 molecule. This is in contrast to the (1-97)IGFBP-3 fragment, where insulin and IGF-I were approximately equipotent in displacing  $^{125}\text{I}$ -IGF-I.

We have shown that IGFBP-3 causes a dose-dependent inhibition of IGF-I-induced IGFIR autophosphorylation in NIH-3T3 cells overexpressing the IGFIR. This inhibition occurs at an 1:1 molar ratio of IGFBP-3 to IGF-I, suggesting an IGF-dependent mechanism of modulation of receptor signaling. The (1-97)  $\text{NH}_2$ -terminal fragment retained the ability to modulate IGF-I binding and signaling via the IGFIR by inhibiting IGF-I-stimulated IGFIR autophosphorylation, albeit at 50-fold higher concentrations than intact IGFBP-3. That this inhibition of IGFIR signaling is largely due to sequestration of IGF-I is strongly supported by the observations that both intact IGFBP-3 and (1-97)IGFBP-3 compete with  $^{125}\text{I}$ -IGF-I binding/cross-linking to the receptor in monolayer affinity cross-linking experiments.

Interestingly, the (98-264) fragment unlike the (1-97)IGFBP-3, failed to show any inhibition of IGFIR signaling, despite its ability to bind IGFs as revealed by *in vitro* binding analysis. The COOH-terminal fragments- (98-264) and (184-264) also failed to compete for  $^{125}\text{I}$ -IGF-I binding and cross-linking to the IGFIR compared to intact IGFBP-3 and the (1-97)  $\text{NH}_2$ -terminal IGFBP-3 fragment. We speculate that the inability of the fragments containing the

COOH-terminal domain of IGFBP-3 to inhibit IGF-I binding to the IGFIR could be due to the following mechanisms: 1) the COOH-terminal fragment binds IGF-I and the entire complex is still capable of binding to and autophosphorylating the IGFIR, implying that the binding site on IGF-I for the receptor and for the carboxy-terminal region of IGFBP-3 are different; 2) the COOH-terminal domain of IGFBP-3 possesses an extracellular matrix binding region and it is possible that in the cellular environment, the fragments containing the COOH-terminal domains are more prone to associate with the cell surface and are not available to sequester IGF-I, especially given their low affinity for IGF. In order to test these hypotheses, the ability of the FLAG-epitope tagged fragments to associate with the cell surface was studied in the presence of IGF-I, with subsequent cross-linking and by analysis of cell lysates on immunoblots probed with anti-IGFBP-3 or M2 anti-FLAG antibody. Our data indicate that the COOH-terminal fragments-(98-264) and (184-264) have the ability to associate to the cell surface in the presence of IGF-I unlike the NH<sub>2</sub> fragment, (1-97)IGFBP-3, which showed no cell-surface association. Further, there was no shift in molecular weight of the cell-surface associated bands and heparin blocked the binding of both intact and the (98-264)IGFBP-3 fragment to the cell surface, ruling out the possibility of interaction of the fragments with any receptor molecule and thereby supporting the second hypothesis. This is in agreement with an earlier study (40), which reported that an IGFBP-3 deletion fragment, lacking the 184-264 region, failed to show any cell-surface association. There are two putative heparin binding motifs in IGFBP-3, located at amino acids 148-153 and 219-226 in the central and carboxyl-terminal regions, respectively, and the carboxyl-terminal motif has been shown to have 4-fold higher affinity for heparin (41). Recently, Bramani *et al*, 1999 (42) have identified two non-basic residues (Gly203 and Gln209) within the extracellular matrix binding region (201-218) in the carboxy-terminal region of IGFBP-5, mutations of which cause 8-10 fold reduction in affinity for human IGF-I. This region is an highly conserved domain in IGFBP-5, -3 and -6 and is known to contain the heparin binding

domain. The authors have proposed that the IGF-I and ECM binding sites partially overlap and heparin binding to the basic amino acids might interfere with IGF-I interaction *in vivo*.

Previous studies with mini-receptor constructs and with isolated domains or proteolytic fragments of the IGF2R (43) urokinase receptor (44), growth hormone receptor (45), talin (46), to name a few have confirmed the involvement of two or more ligand contact regions. Similarly, in the case of IGFBP-3, it appears that the IGF and insulin binding domains are bipartite and possibly overlapping. In our biological system, the stoichiometry of IGFBP-3 binding to IGF-I seems to be 1:1. We postulate that both NH<sub>2</sub>- and COOH-terminal domains have residues that are capable of binding IGF-I and insulin with low affinity. However, there is simultaneous interaction of the two "half sites" in intact IGFBP-3, which creates an high-affinity IGF binding site on the molecule. Simultaneously, this interaction leads to a markedly reduced ability of intact IGFBP-3 to bind insulin, possibly due to masking of the residues which interact with insulin, as a result of tertiary conformational change (21). With respect to IGF binding to IGFBP-3, it is unclear whether the NH<sub>2</sub>- and COOH-terminal domains contribute equally. We predict the presence of functional residues in the NH<sub>2</sub>-terminus (1-97) and COOH-terminus (149-264), that confer high-affinity by cooperative or conformational changes; the structural residues that mediate the necessary non-covalent interactions may reside in the NH<sub>2</sub>-, intermediate or COOH-terminus of the IGFBP-3 molecule. Alternatively, given the striking similarity of the NH<sub>2</sub>-terminal domains and the fact that this region is encoded by a single exon in all of the classical IGFBPs and the IGFBP<sub>r</sub>Ps (low affinity IGF binders), it is possible that the NH<sub>2</sub>-terminus is the critical functional component involved in binding IGFs, and that conformational effects imposed on the NH<sub>2</sub>-terminus by the COOH-terminal domain are required for high-affinity binding.

In summary, the present study, along with previous work from our and other laboratories, clearly demonstrate the ability of the IGFBP-3 amino-terminal fragment to bind IGF and insulin and to inhibit IGFIR and insulin receptor autophosphorylation (21,22), revealing that this 16 kDa fragment may be capable of both IGF-I-dependent and IGF-independent roles in modulating cell growth. However, the carboxyl-terminal fragments, which also have the ability to bind both IGF-I and insulin *in vitro*, fail to prevent binding of either IGF-I or insulin (data not shown) to their respective receptors, due to the tendency of these fragments for cell surface association via the heparin binding domain. Also intriguing is the identification of a thyroglobulin-like motif in the COOH-terminal regions of IGFBP superfamily, which has also been found in the superfamily of protein inhibitors of cysteine proteinases (47,48). Whether this highly conserved thyroglobulin type-I element indeed acts as an inhibitor of cysteine proteinases in these proteins, remains to be established. The intermediate region of IGFBP-3 does not appear to bind IGFs or insulin and its role in high-affinity binding to IGFs is probably related to its ability to promote proper tertiary structure and optimal interactions between the amino- and carboxy-terminal residues. Further, it has been demonstrated that the intermediate region of IGFBP-3 is involved in the specific interaction between IGFBP-3 and its putative cell-surface receptor (26). Taken together, it is tempting to speculate that various forms of IGFBP-3 fragments resulting from proteolysis by IGFBP-3 specific proteases will have different effects on the IGF-IGFIR axis, as well as potential IGF-independent actions.

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## Footnotes

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\* Abbreviations used in the text are: IGF, insulin-like growth factor; IGFIR, insulin-like growth factor I receptor; IGFBP, insulin-like growth factor binding protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; BSA, bovine serum albumin; DSS, disuccinimidyl suberate; ECM, extracellular matrix.

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## FIGURE LEGENDS

**Figure 1. Expression of FLAG-epitope tagged human IGFBP-3 and its fragments.** A, The cDNA for preparation of the IGFBP-3 fragments were synthesized by a series of PCR reactions using the human IGFBP-3 cDNA as template and incorporating sequence encoding a COOH-terminal FLAG epitope tag. The *dark boxes* (1-87) represent the conserved NH<sub>2</sub>-terminal region, the *diamond striped boxes* (183-264), the conserved COOH-terminal region and the *square striped boxes* (88-182) represent the variable intermediate region of IGFBP-3. The predicted molecular weights are based on the amino acid composition of the proteins. B, Protein expression was analyzed by immunoblotting using the M2 monoclonal antibody under reducing conditions or the IGFBP-3 monoclonal antibody under non-reducing conditions, coupled to ECL.

**Figure 2. IGF binding analysis.** A, <sup>125</sup>I-IGF-I affinity cross-linking: IGFBP-3 proteolytic fragments were incubated with <sup>125</sup>I-IGF-I (1x10<sup>5</sup> cpm) in a 100 µl volume for 16 h at 4°C and then cross-linked with 0.5 mM DSS for 15 min at 4°C. The affinity labeled fragments were separated on a 15% SDS polyacrylamide gel under reducing conditions. An autoradiogram of the gel is shown. Values associated with the arrows indicate the calculated molecular weights of the major radioactive species. B, Competitive <sup>125</sup>I-IGF-I affinity cross-linking: IGFBP-3 fragments (50 nM) equivalent to 5 pmoles, were incubated with radiolabeled IGF-I in the presence or absence of the indicated concentrations of unlabeled IGF-I or insulin. Cross-linking was then done with DSS, followed by SDS-PAGE under reducing conditions. The autoradiogram shown is representative of three replicates. The arrows indicate the major radioactive species. C, Quantitative analysis of radiolabeled <sup>125</sup>I-IGF-II displacement from IGFBP-3 fragments: The gels



shown in Fig. 2B were densitometrically analyzed for quantitative estimation of the radioactivity associated with the individual bands, except in the case of 98-264 displacement with cold IGF-I, in which affinity cross-linking was done with lower concentrations of cold IGF-I to construct the displacement curve. The data have been expressed as a percentage of maximal band intensity.

**Figure 3. IGF-I western ligand blot.** IGFBP-3 and the proteolytic fragments (indicated concentrations), were electrophoresed on 12% SDS-PAGE under non-reducing conditions. After electroblotting to nitrocellulose, the blots were incubated with a mixture of  $^{125}\text{I}$ -IGF-I and  $^{125}\text{I}$ -IGF-II. Values associated with the arrows indicate the calculated molecular weights of the major radioactive species.

**Figure 4. Insulin Binding analysis.** A,  $^{125}\text{I}$ -insulin affinity cross-linking: IGFBP-3 proteolytic fragments were incubated with  $^{125}\text{I}$ -Insulin ( $1 \times 10^5$  cpm) in a 100  $\mu\text{l}$  volume for 16 h at  $4^\circ\text{C}$  and then cross-linked with 0.5 mM DSS for 15 min at  $4^\circ\text{C}$ . The affinity labeled fragments were separated on a 15% SDS polyacrylamide gel. An autoradiogram of the gel is shown, which is representative of three replicates. Values associated with the arrows indicate the calculated molecular weights of the major radioactive species. B, Competitive  $^{125}\text{I}$ -insulin affinity cross-linking: IGFBP-3 and the proteolytic fragments (50 nM) equivalent to 5 pmoles, were incubated with radiolabeled insulin in the presence or absence of the indicated concentrations of unlabeled insulin or IGF-I. Cross-linking was then done with DSS, followed by SDS-PAGE. Autoradiogram of the dried gel shown. The arrows indicate the major radioactive species. C, Quantitative analysis of radiolabeled  $^{125}\text{I}$ -Insulin displacement from IGFBP-3 fragments: The gels shown in Fig. 4B were densitometrically analyzed for quantitative estimation of the radioactivity

associated with the individual bands. The data have been expressed as a percentage of maximal band intensity.

**Figure 5. Monolayer affinity cross-linking with  $^{125}\text{I}$ -IGF-I.** A,  $^{125}\text{I}$ -IGF-I was preincubated at 4°C in the presence or absence of unlabeled IGF-I (100 nM), IGFBP-3 (30 nM) or fragments (250 nM) and then these treatments were added to confluent monolayers of NIH-3T3-IGFIR cells for 3 h at 15°C. After washing, the cells were cross-linked and cell lysates were run on a 6% SDS-PAGE gel. The arrow indicates the IGFIR species cross-linked to radiolabeled IGF-I. B, A set of the same cell lysates were run on a 15% SDS-PAGE under reduced conditions and immunoblotted with M2 anti-FLAG monoclonal antibody.

**Figure 6. Effect of heparin on cell surface association of IGFBP-3 and its fragments.** Confluent NIH-3T3-IGFIR cells were treated with either peptides alone- lane 1: untreated cells, lane 2: IGFBP-3, lane 5: (98-264)IGFBP-3, lane 8:(1-97)IGFBP-3; or with peptides preincubated with heparin for 1 h at 4°C-lane 4: IGFBP-3+heparin, lane 7: (98-264)IGFBP-3+heparin; or the cells were first treated with heparin for 1 h, washed and then the following peptides were added-lane 3: IGFBP-3, lane 6: (98-264)IGFBP-3. All the treatments were carried out for 3 h at 15°C. After washing, the cells were cross-linked and cell lysates were run on a 15% SDS-PAGE and immunoblotted with anti-IGFBP-3 monoclonal antibody. The arrows indicate the cell surface associated species.

**Figure 7. IGFIR autophosphorylation assay.** A, Confluent NIH-3T3-IGFIR cells stably transfected with the human IGFIR cDNA were exposed for either 5 min or 10 min to 1, 7, and 14

nM IGF-I peptide. The reaction was quenched by solubilization buffer and the solubilized proteins were separated by 7.5% SDS-PAGE under reducing conditions and visualized by immunoblot analysis using anti-phosphotyrosine monoclonal antibody. The arrow indicates the 95 kDa beta subunit of IGFIR. B, Confluent NIH-3T3-IGFIR cells were exposed for 5 min to 50 ng/ml IGF-I, which had been preincubated with IGFBP-3, 1-97 or 98-264 proteolytic fragment for 2 h at 4°C. The solubilized proteins were separated by 7.5% SDS-PAGE under reducing conditions and visualized by immunoblot analysis using anti-phosphotyrosine monoclonal antibody. The arrows indicate the 95 kDa beta subunit of IGFIR in each immunoblot. C, The specific 95 kDa bands representing the phosphorylated  $\beta$  subunit of the IGFIR and the 116 kDa non-specific bands in the gels shown in Fig. 7B and in other replicate experiments (n=2-4) were densitometrically analyzed. The ratio of the two band intensities was used to normalize and quantify the percentage of maximal IGF-I-induced IGFIR autophosphorylation detected in the presence of intact IGFBP-3 and its fragments.

## FIGURE LEGENDS

**Figure 1. Expression of FLAG-epitope tagged human IGFBP-3 and its fragments.** A, The cDNA for preparation of the IGFBP-3 fragments were synthesized by a series of PCR reactions using the human IGFBP-3 cDNA as template and incorporating sequence encoding a COOH-terminal FLAG epitope tag. The *dark boxes* (1-87) represent the conserved NH<sub>2</sub>-terminal region, the *diamond striped boxes* (183-264), the conserved COOH-terminal region and the *square striped boxes* (88-182) represent the variable intermediate region of IGFBP-3. The predicted molecular weights are based on the amino acid composition of the proteins. B, Protein expression was analyzed by immunoblotting using the M2 monoclonal antibody under reducing conditions or the IGFBP-3 monoclonal antibody under non-reducing conditions, coupled to ECL.

**Figure 2. IGF binding analysis.** A, <sup>125</sup>I-IGF-I affinity cross-linking: IGFBP-3 proteolytic fragments were incubated with <sup>125</sup>I-IGF-I (1x10<sup>5</sup> cpm) in a 100 µl volume for 16 h at 4°C and then cross-linked with 0.5 mM DSS for 15 min at 4°C. The affinity labeled fragments were separated on a 15% SDS polyacrylamide gel under reducing conditions. An autoradiogram of the gel is shown. Values associated with the arrows indicate the calculated molecular weights of the major radioactive species. B, Competitive <sup>125</sup>I-IGF-I affinity cross-linking: IGFBP-3 fragments (50 nM) equivalent to 5 pmoles, were incubated with radiolabeled IGF-I in the presence or absence of the indicated concentrations of unlabeled IGF-I or insulin. Cross-linking was then done with DSS, followed by SDS-PAGE under reducing conditions. The autoradiogram shown is representative of three replicates. The arrows indicate the major radioactive species. C,

Quantitative analysis of radiolabeled  $^{125}\text{I}$ -IGF-II displacement from IGFBP-3 fragments: The gels shown in Fig. 2B were densitometrically analyzed for quantitative estimation of the radioactivity associated with the individual bands, except in the case of 98-264 displacement with cold IGF-I, in which affinity cross-linking was done with lower concentrations of cold IGF-I to construct the displacement curve. The data have been expressed as a percentage of maximal band intensity.

**Figure 3. IGF-I western ligand blot.** IGFBP-3 and the proteolytic fragments (indicated concentrations), were electrophoresed on 12% SDS-PAGE under non-reducing conditions. After electroblotting to nitrocellulose, the blots were incubated with a mixture of  $^{125}\text{I}$ -IGF-I and  $^{125}\text{I}$ -IGF-II. Values associated with the arrows indicate the calculated molecular weights of the major radioactive species.

**Figure 4. Insulin Binding analysis.** A,  $^{125}\text{I}$ -insulin affinity cross-linking: IGFBP-3 proteolytic fragments were incubated with  $^{125}\text{I}$ -Insulin ( $1 \times 10^5$  cpm) in a 100  $\mu\text{l}$  volume for 16 h at  $4^\circ\text{C}$  and then cross-linked with 0.5 mM DSS for 15 min at  $4^\circ\text{C}$ . The affinity labeled fragments were separated on a 15% SDS polyacrylamide gel. An autoradiogram of the gel is shown, which is representative of three replicates. Values associated with the arrows indicate the calculated molecular weights of the major radioactive species. B, Competitive  $^{125}\text{I}$ -insulin affinity cross-linking: IGFBP-3 and the proteolytic fragments (50 nM) equivalent to 5 pmoles, were incubated with radiolabeled insulin in the presence or absence of the indicated concentrations of unlabeled insulin or IGF-I. Cross-linking was then done with DSS, followed by SDS-PAGE. Autoradiogram of the dried gel shown. The arrows indicate the major radioactive species. C, Quantitative analysis of radiolabeled  $^{125}\text{I}$ -Insulin displacement from IGFBP-3 fragments: The gels shown in

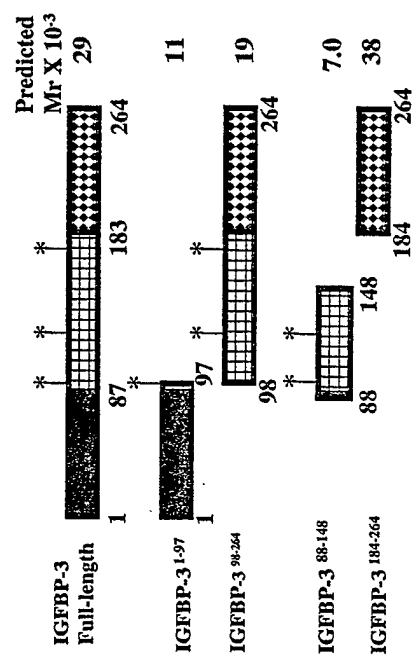
Fig. 4B were densitometrically analyzed for quantitative estimation of the radioactivity associated with the individual bands. The data have been expressed as a percentage of maximal band intensity.

**Figure 5. Monolayer affinity cross-linking with  $^{125}\text{I}$ -IGF-I.** A,  $^{125}\text{I}$ -IGF-I was preincubated at 4°C in the presence or absence of unlabeled IGF-I (100 nM), IGFBP-3 (30 nM) or fragments (250 nM) and then these treatments were added to confluent monolayers of NIH-3T3-IGFIR cells for 3 h at 15°C. After washing, the cells were cross-linked and cell lysates were run on a 6% SDS-PAGE gel. The arrow indicates the IGFIR species cross-linked to radiolabeled IGF-I. B, A set of the same cell lysates were run on a 15% SDS-PAGE under reduced conditions and immunoblotted with M2 anti-FLAG monoclonal antibody.

**Figure 6. Effect of heparin on cell surface association of IGFBP-3 and its fragments.** Confluent NIH-3T3-IGFIR cells were treated with either peptides alone- lane 1: untreated cells, lane 2: IGFBP-3, lane 5: (98-264)IGFBP-3, lane 8:(1-97)IGFBP-3; or with peptides preincubated with heparin for 1 h at 4°C-lane 4: IGFBP-3+heparin, lane 7: (98-264)IGFBP-3+heparin; or the cells were first treated with heparin for 1 h, washed and then the following peptides were added-lane 3: IGFBP-3, lane 6: (98-264)IGFBP-3. All the treatments were carried out for 3 h at 15°C. After washing, the cells were cross-linked and cell lysates were run on a 15% SDS-PAGE and immunoblotted with anti-IGFBP-3 monoclonal antibody. The arrows indicate the cell surface associated species.

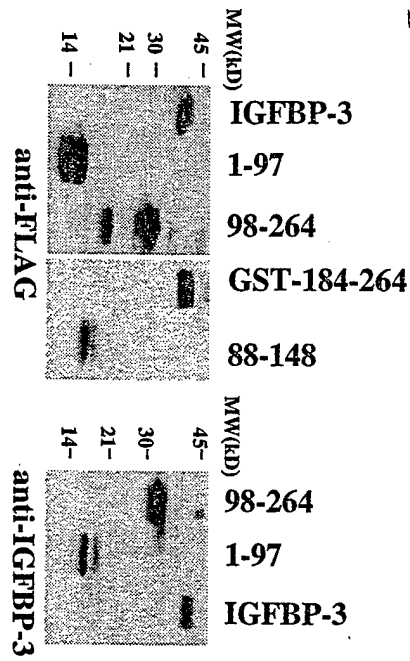
**Figure 7. IGFIR autophosphorylation assay.** A, Confluent NIH-3T3-IGFIR cells stably transfected with the human IGFIR cDNA were exposed for either 5 min or 10 min to 1, 7, and 14 nM IGF-I peptide. The reaction was quenched by solubilization buffer and the solubilized proteins were separated by 7.5% SDS-PAGE under reducing conditions and visualized by immunoblot analysis using anti-phosphotyrosine monoclonal antibody. The arrow indicates the 95 kDa beta subunit of IGFIR. B, Confluent NIH-3T3-IGFIR cells were exposed for 5 min to 50 ng/ml IGF-I, which had been preincubated with IGFBP-3, 1-97 or 98-264 proteolytic fragment for 2 h at 4°C. The solubilized proteins were separated by 7.5% SDS-PAGE under reducing conditions and visualized by immunoblot analysis using anti-phosphotyrosine monoclonal antibody. The arrows indicate the 95 kDa beta subunit of IGFIR in each immunoblot. C, The specific 95 kDa bands representing the phosphorylated  $\beta$  subunit of the IGFIR and the 116 kDa non-specific bands in the gels shown in Fig. 7B and in other replicate experiments (n=2-4) were densitometrically analyzed. The ratio of the two band intensities was used to normalize and quantify the percentage of maximal IGF-I-induced IGFIR autophosphorylation detected in the presence of intact IGFBP-3 and its fragments.

**A**

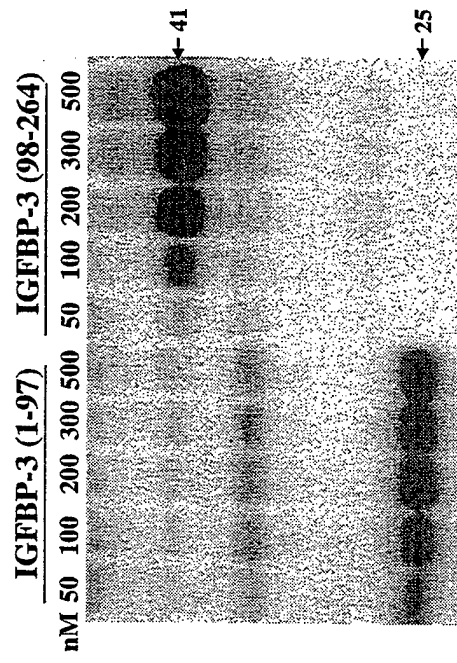




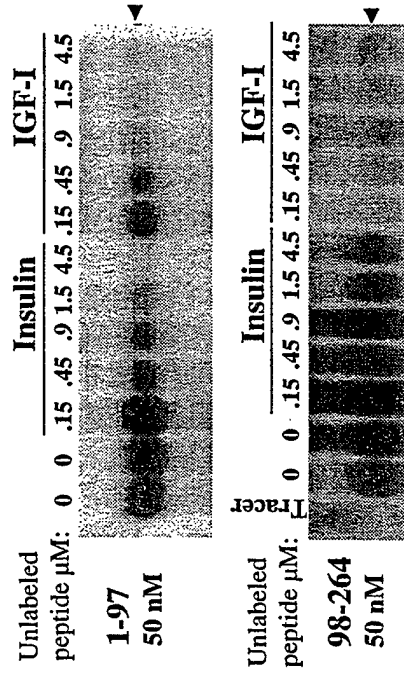
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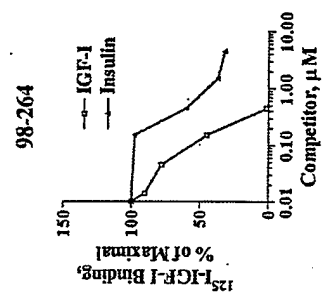
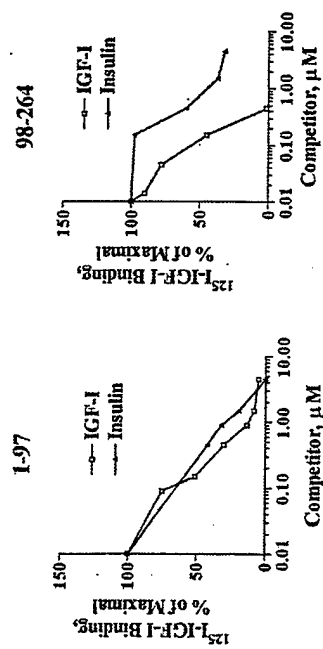
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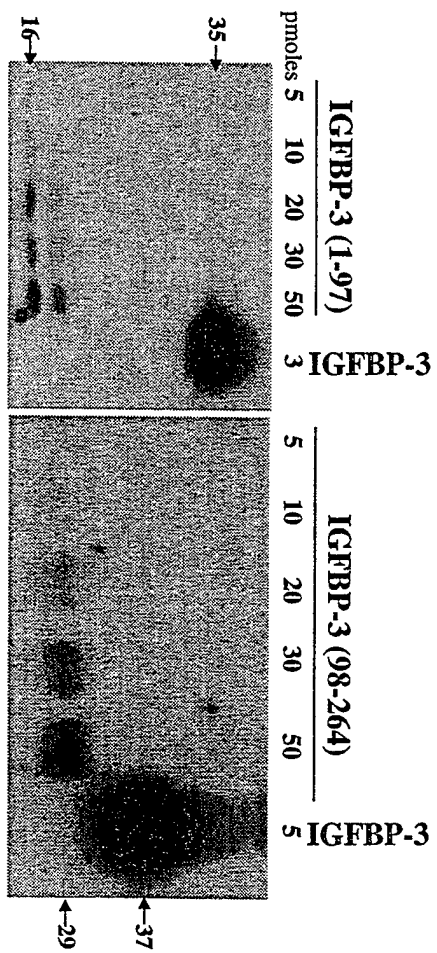


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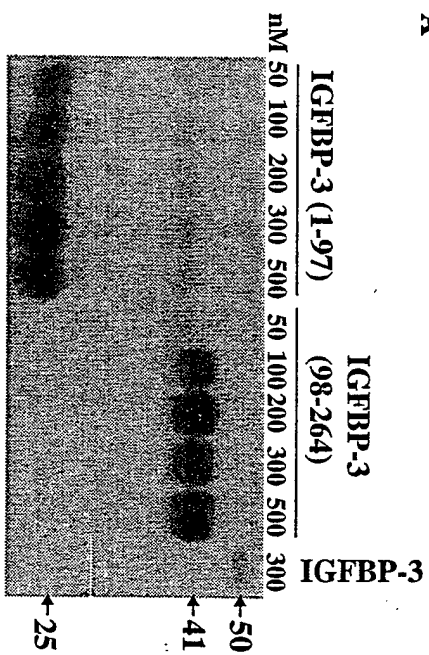


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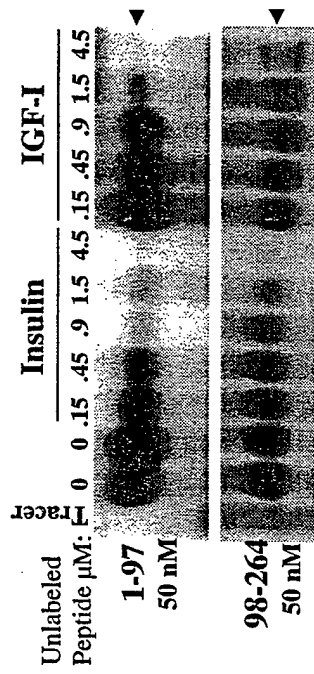




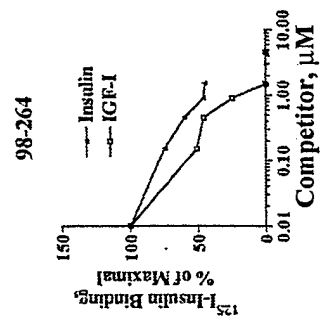
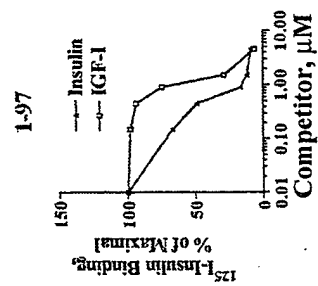
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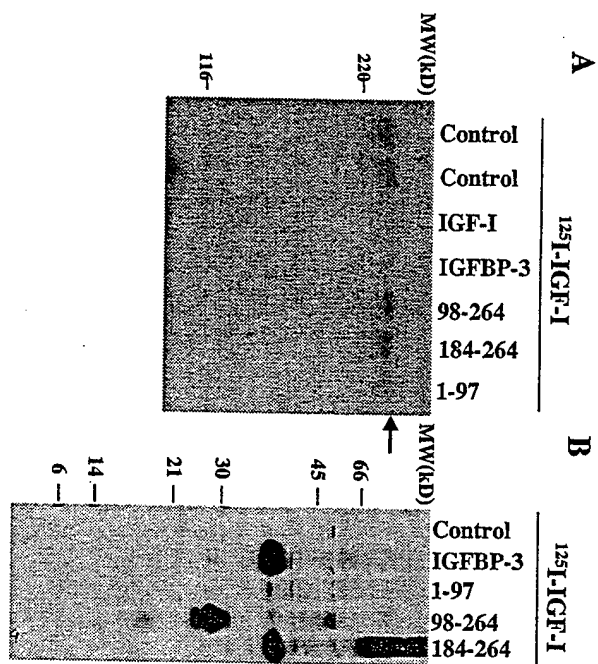
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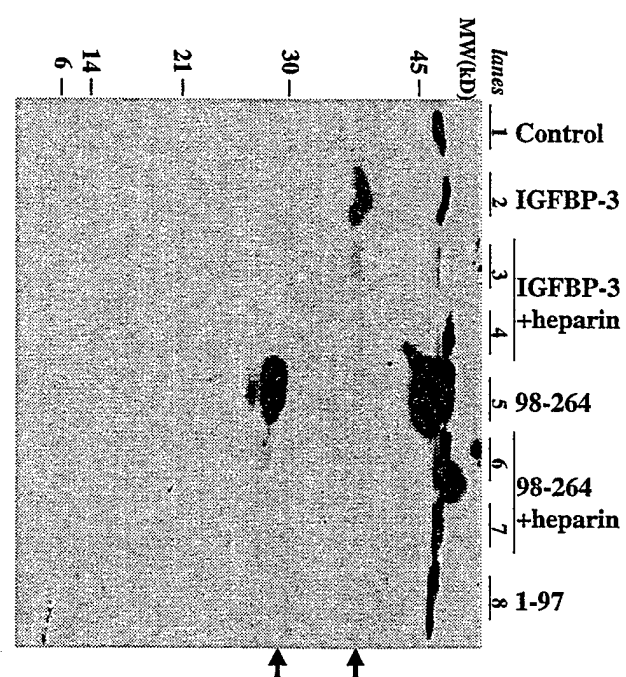


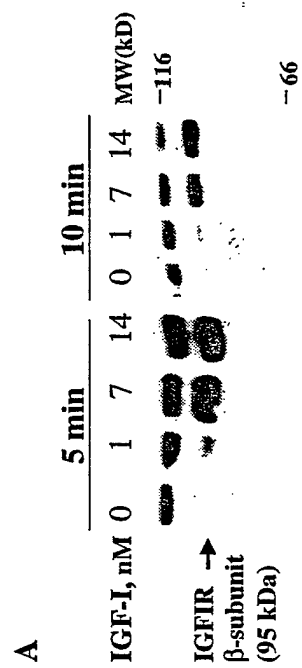
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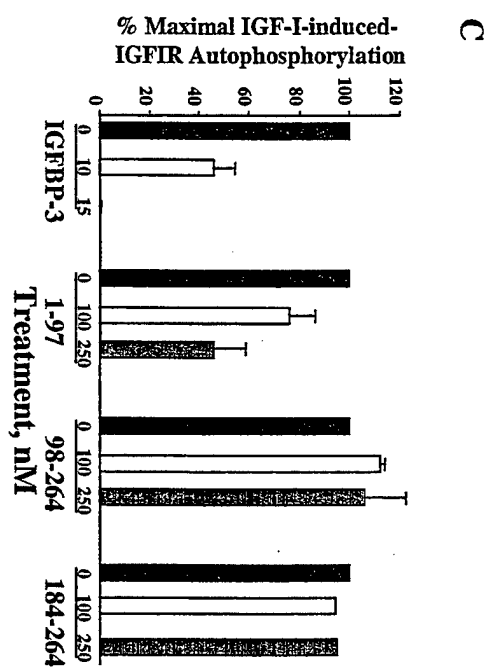












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S U P P L E M E N T   T O   P E D I A T R I C S

*National Cooperative Growth Study:  
Guidance in Growth*

*Proceedings of the National Cooperative  
Growth Study Twelfth Annual  
Investigators Meeting, New Orleans, LA  
October 8-11, 1998*

*Organized and hosted by Arkansas Children's Hospital, a major  
teaching affiliate of the University of Arkansas for Medical  
Sciences*

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# The Insulin-like Growth Factor Binding Protein Superfamily: New Perspectives

Ron G. Rosenfeld, MD; Vivian Hwa, PhD; Lisa Wilson, BS; Abel Lopez-Bermejo, MD; Caroline Buckway, MD; Christine Burren, MD; Won Kyu Choi, MD; Gayathri Devi, PhD; Angela Ingermann, MS; Donna Graham, BS; Giuseppi Minniti, MD; Anna Spagnoli, MD; and Youngman Oh, PhD

**ABSTRACT.** The insulin-like growth factor (IGF) binding proteins (IGFBPs) were initially identified as carrier proteins for IGF-I and IGF-II in a variety of biologic fluids. Their presumed function was to protect IGF peptides from degradation and clearance, increase the half-life of the IGFs, and deliver them to appropriate tissue receptors. The concept of IGFBPs as simple carrier proteins has been complicated, however, by a number of observations: 1) the six IGFBPs vary in their tissue expression and their regulation by other hormones and growth factors; 2) the IGFBPs are subjected to proteolytic degradation, thereby altering their affinities for the IGFs; 3) IGFBP-3 and IGFBP-5, in addition to binding IGFs, also can associate with an acid-labile subunit, thereby increasing further the half-life of the IGFs; 4) in addition to modifying the access of IGF peptides to IGF and insulin receptors, several of the IGFBPs may be capable of increasing IGF action; 5) some of the IGFBPs may be capable of IGF-independent regulation of cell growth; 6) some of the IGFBPs are associated with cell membranes or possibly with membrane receptors; and 7) some of the IGFBPs have nuclear recognition sites and may be found within the nucleus. Additionally, a number of cDNAs identified recently have been found to encode proteins that bind IGFs, but with substantially lower affinities than is the case with IGFBPs. The N-terminal regions of the predicted proteins are structurally homologous to the classic IGFBPs, with conservation of the cysteine-rich region. These observations suggest that these low-affinity binders are members of an IGFBP superfamily, capable of regulating cell growth by both IGF-dependent and IGF-independent mechanisms. *Pediatrics* 1999;104:1018-1020; *insulin-like growth factor, insulin-like growth factor binding proteins*.

**ABBREVIATIONS.** IGF, insulin-like growth factor; IGFBP, IGF binding protein; TAF, tumor adhesion factor; PSF, prostacyclin-stimulating factor; IGFBP-rP, IGFBP-related protein.

In serum and other biologic fluids, the insulin-like growth factors (IGFs) are bound to members of a family of high-affinity IGF binding proteins (IGFBPs).<sup>1-3</sup> The affinity of these IGFBPs for IGF-I and IGF-II is sufficiently high ( $K_d$ ,  $10^{-11}$  to  $10^{-10}$  mol/L) to ensure that virtually all circulating IGF is

bound to IGFBPs. Six members of the IGFBP family have been identified, all encoded by distinct genes located on four separate chromosomes (Table 1). The IGFBPs were identified originally as carrier proteins for the IGFs in serum, but the existence of six distinct IGFBPs in all mammalian species studied to date, each encoded by separate, independent genes and regulated in a tissue-specific manner, has suggested that the biologic functions of the IGFBPs may be more complex than originally believed.

The physiologic roles of the IGFBPs may be divided into IGF-dependent and IGF-independent activities.<sup>4</sup> The former refers to the ability of IGFBPs to 1) transport IGFs in plasma and other biologic fluids; 2) increase the half-lives of IGF peptides, typically from minutes to hours; and 3) modulate the access of IGF ligands to their receptors, thereby regulating IGF action. The latter, IGF-independent activities, refers to the ability of IGFBPs to regulate cell growth, migration, or metabolism in manners that are independent of IGF action, presumably through direct interactions of the IGFBPs with cellular receptors or "interacting proteins."<sup>5</sup> The observation that certain IGFBPs are proteolyzed under a variety of physiologic and pathologic conditions has complicated additionally issues related to IGFBP action. Proteolysis of IGFBPs first was shown in human pregnancy serum, in which circulating IGFBP-3 was found primarily in low-molecular-weight forms.<sup>6,7</sup> These proteolytic fragments of IGFBP-3 were shown to bind IGF with lower affinity, potentially altering the release of IGF by IGFBPs to target receptors.

Subsequent studies have shown that proteolysis is not restricted to IGFBP-3 and that limited proteolysis of IGFBPs 2 through 5 may 1) alter the affinity of the IGFBP fragment for IGF, 2) modulate the release of IGF from IGFBPs to target receptors, and 3) produce IGFBP fragments that are capable of direct stimulatory or inhibitory action.

The multiplicity of potential biologic actions of IGFBPs and IGFBP fragments is, perhaps, best understood by recognizing that the IGFBP family is part of a protein superfamily, the members of which all preserve the cysteine-rich N-terminal domain that is characteristic of the six high-affinity IGFBPs (Fig 1).<sup>8-10</sup> In addition to this cysteine-rich N-terminal domain, IGFBPs 1 through 6 also share homology in the C-terminal region, but vary in the midregion of the protein. In the N-terminal region, IGFBPs 1 through 5 conserve all 12 cysteines and IGFBP-6 has 10 of the 12 cysteines. Additionally, within the N-terminal region, IGFBPs 1 through 5 conserve a GCGCCxxC motif and IGFBP-6 substitutes a GCAE-

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TABLE 1. Characteristics of the Human IGFBP Superfamily

Protein	Molecular Weight	Number of Amino Acids	Number of Cysteines	N-linked Glycosylation	Chromosome	mRNA (kb)
High-affinity IGFBPs						
IGFBP-1	25,271	234	18	No	7p	1.6
IGFBP-2	31,355	289	18	No	2q	1.5
IGFBP-3	28,717	264	18	Yes	7p	2.4
IGFBP-4	25,957	237	20	Yes	17q	1.7
IGFBP-5	28,553	252	18	No	2q	1.7,6.0
IGFBP-6	22,847	216	16	No	12	1.1
Low-affinity IGFBPs and IGFBP-rPs*						
IGFBP proteolytic fragments						
IGFBP-rP1	26,400	251	18	Yes	4q	1.1
IGFBP-rP2	35,500	349 (prepeptide)	39	Yes	6q	2.4
IGFBP-rP3	36,000	357 (prepeptide)	41	?No	8q	2.4
IGFBP-rP4	39,500	381 (prepeptide)	35	?No	1p	2.5,4.0

\* Nomenclature for the IGFBP-rPs is given in Table 2.

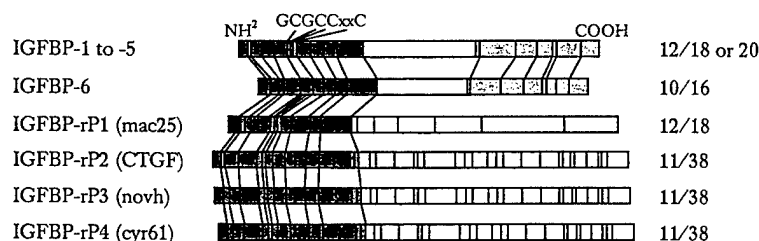


Fig 1. Schematic diagram of the IGFBP superfamily, consisting of IGFBPs 1 through 6 and IGFBP-rPs 1 through 4. The conserved N-terminal domains are shown as dark shaded bars. The conserved C-terminal domains in IGFBPs 1 through 6 are shown as light shaded bars. Nonconserved regions are indicated by open bars. Conserved cysteines are indicated by diagonal lines connecting the bars; other cysteines are indicated by vertical lines. The numbers to the right of the bars indicate the number of N-terminal conserved cysteines/total number of cysteines in the molecule.

AEGC motif (thereby accounting for the two "missing" cysteines). The significance of this highly conserved motif is not certain, and its role in the binding of IGF ligand is not clear. The IGFBPs, thus, may be considered modular proteins, with two conserved modules and one variable one. This concept is supported by the preservation of even numbers of cysteines in the N (10 or 12 cysteines) and the C (6 cysteines) regions, suggesting that covalent disulfide bonds form within each discrete module, leaving no free cysteines to interact between the two domains.

mac25 originally was identified as a cDNA that is expressed in normal leptomeninges, but not in certain meningiomas<sup>11</sup>; similar subtractive hybridization studies in mammary tissue subsequently resulted in the identification of the same cDNA in senescent normal mammary tissue but not in some breast carcinomas.<sup>12</sup> The deduced protein was predicted to conserve the N-terminal domain of the IGFBPs, including all 12 cysteines and the G/ACGC-CxxC motif. Oh et al<sup>18</sup> synthesized the protein in a baculovirus expression system and found that mac25 bound IGF-I and IGF-II, but with considerably lower affinity than that observed with IGFBPs 1 through 6. This ability of mac25 protein to bind IGFs (albeit with less affinity) was consistent with the observation that N-terminal fragments of IGFBP-3 (both naturally occurring proteolytic fragments and synthetic peptides) retain the ability to bind IGF.<sup>13</sup> Based on the structural homology with the IGFBPs and the

conservation of the ability to bind IGFs, the mac25 protein was provisionally named IGFBP-7.<sup>8</sup> The same protein had been purified independently from a human bladder carcinoma cell line and named tumor adhesion factor (TAF) and from human diploid fibroblasts and named prostacyclin-stimulating factor (PSF) (Table 2).<sup>14</sup>

TABLE 2. Proposed Nomenclature for the IGFBP Superfamily

High-affinity IGFBPs	
IGFBP-1	
IGFBP-2	
IGFBP-3	
IGFBP-4	
IGFBP-5	
IGFBP-6	
Low-affinity IGFBPs and IGFBP-rPs	
IGFBP proteolytic fragments	
IGFBP-related proteins (IGFBP-rPs)	
Published name	IGFBP-rP
mac25	IGFBP-rP1
Tumor adhesion factor (TAF)	
Prostacyclin-stimulating factor (PSF)	
IGFBP-7 (provisional)	
Connective tissue growth factor (CTGF)	IGFBP-rP2
IGFBP-8 (provisional)	
Nephroblastoma overexpressed gene (novH)	IGFBP-rP3 (provisional)
cyr61	IGFBP-rP4 (provisional)



The growing understanding of the importance of the cysteine-rich N-terminal domain in IGF binding and its preservation in other modular proteins, such as mac25, led to a closer examination of the CCN family of proteins (C, connective tissue growth factor; C, CYR61, a growth factor-inducible immediate-early gene; N, NOVH [neuroblastoma overexpression gene]). Like the high-affinity IGFBPs and mac25, the CCN family members are modular proteins, with the conserved cysteine-rich N-terminal domain, including the GCG/SCCxxC motif.<sup>15</sup> Connective tissue growth factor and novH proteins now have been synthesized in baculovirus expression systems and, as with mac25, bind IGF-I and -II with low affinity.<sup>9</sup>

The high-affinity IGFBPs, mac25, and the CCN family thus are all modular proteins that conserve an N-terminal, cysteine-rich domain that appears to confer the ability of each protein to bind IGFs. The ability of IGFBPs 1 through 6 to bind IGF-I and -II with particularly high-affinity results, presumably, from the interaction of the N-terminal and the C-terminal domains; the conserved C-terminal domain is unique to the high-affinity IGFBPs. The crucial role of the cysteine-rich N-terminal domain is underscored by the fact that it is encoded by a single exon in genes for all these proteins; this strongly suggests that exon shuffling, with the dissemination of the DNA sequence that encodes the N-terminal domain, resulted in the distribution of this exon among various genes by a series of DNA recombinational events. The preservation of the cysteine-rich N-terminal module and the ability to bind IGFs have led to the proposal of an IGFBP superfamily, encompassing the high-affinity IGFBPs and the low-affinity IGFBP-related proteins (IGFBP-rPs) (Table 2; Fig 1).<sup>16</sup>

In addition to its role in binding IGFs, the cysteine-rich N-terminal domain rather unexpectedly has an affinity for insulin.<sup>17</sup> Indeed, IGFBPs 1 through 6 are all capable of binding insulin, but with an affinity substantially lower than for IGF-I or IGF-II. On the other hand, IGFBP-rP1 appears to be capable of binding insulin with an affinity at least as great as its affinity for IGF peptides. Similarly, the cysteine-rich N-terminal domain of IGFBP-3 has less affinity for IGFs than does intact IGFBP-3, but greater affinity for insulin. Thus it is apparent that the secondary and tertiary structures of the IGFBPs, resulting from interactions between the N-terminal and the C-terminal domains, confer the capability of high-affinity binding of IGFs. Disrupting this tertiary structure, by a reduction of disulfide bonds or by proteolysis, results in a sharp reduction in affinity for IGFs, accompanied by an increased affinity for insulin.

The ability of the high-affinity IGFBPs to bind IGFs is clearly central to their IGF-dependent actions. Whether the binding of insulin by IGFBP, IGFBP proteolytic fragments, or IGFBP-rPs has physiologic significance remains uncertain at this time. It appears highly likely, however, that the preservation of the cysteine-rich N-terminal domain throughout the IGFBP superfamily is crucial to the IGF-dependent and IGF-independent ac-

tions of all these proteins. It is expected that additional investigation into structure-function relations among the members of the IGFBP superfamily will lead to further understanding of the importance of this highly conserved domain.

## ACKNOWLEDGMENTS

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**TITLE:**

CHARACTERIZATION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-RELATED PROTEIN (IGFBP-rP) 2 AND 3 IN HUMAN PROSTATE EPITHELIAL CELLS. POTENTIAL ROLES OF IGFBP-rP1 AND 2 DURING SENESCENCE OF THE PROSTATIC EPITHELIUM

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**KEY WORDS**

IGFBP-rP1, IGFBP-rP2, IGFBP-rP3, MAC25, Angiomodulin, PSF, T1A12, CTGF, NOV, Prostate cells, Prostate cancer

# **Characterization of Insulin-Like Growth Factor Binding Protein-Related Protein (IGFBP-rP) 2 and 3 in Human Prostate Epithelial Cells. Potential roles of IGFBP-rP1 and 2 during senescence of the Prostatic Epithelium.**

## **Abstract**

IGFBP-rPs are newly described cysteine-rich proteins that share significant amino-terminal structural similarity with the conventional IGFBPs and are involved in a diversity of biological functions, including growth regulation. IGFBP-rP1 (MAC25/TAF/PSF) is a potential tumor suppressor gene that is differentially expressed in meningiomas, mammary and prostatic cancers, versus corresponding normal and senescent tissues. We have previously shown that IGFBP-rP1 is preferentially produced by primary cultures of prostate epithelial cells (HPEC) and by poorly tumorigenic P69 cells, compared to the cancerous prostatic LNCaP, DU145, PC-3 and M12 cells. We now show that IGFBP-rP1 also increases during senescence of HPECs.

IGFBP-rP2 (CTGF), a modulator of growth for both fibroblasts and endothelial cells, was detected in most of the normal and malignant prostatic epithelial cells tested, with a marked up-regulation of IGFBP-rP2 during senescence of HPEC. Moreover, IGFBP-rP2 noticeably increased in

response to transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) and all-*trans* retinoic acid (atRA) in HPEC and PC-3 cells, and decreased upon treatment with IGF-I in HPEC.

IGFBP-rP3 (NOV), the protein product of the *NOV* proto-oncogene, was not detected in HPEC, but was expressed in the tumorigenic DU145 and PC-3 cells. It was also synthesized by the SV40 T-antigen transformed P69 and M12 cells, where it was down-regulated by atRA.

These observations suggest biological roles of IGFBP-rPs in the human prostate. IGFBP-rP1 and IGFBP-rP2 are likely to negatively regulate growth, as they increase during senescence of the prostate epithelium and in response to growth inhibitors (TGF $\beta$ 1 and atRA). Although the data collected on IGFBP-rP3 in prostate are modest, its role as a growth stimulator and/or protooncogene is supported by its preferential expression in cancerous cells and its down-regulation by atRA.

## Introduction

The IGF system is composed of two ligands, IGF-I and IGF-II, six IGFBPs, IGFBP-1 to -6, and two receptors, the type 1 and type 2 IGF receptors (1). Recently, the IGFBP family of proteins has been expanded to include additional members that share significant structural similarities, the so-called IGFBP-rPs (2). Thus, the IGFBP Superfamily comprises the six conventional IGFBPs, which show high affinity for IGFs, and at least ten IGFBP-rPs, which not only share the conserved amino-terminal domain of the IGFBPs, but also show some degree of affinity for IGFs and insulin.

The IGF system has been extensively studied in the human prostate, where it has been shown to be necessary for normal growth of prostate epithelial and stromal cells (3). Furthermore, abnormalities in the IGF system have been identified in prostate disease, including prostate hyperplasia and cancer. In this respect, prostate cancer growth appears to be poorly dependent on IGFs since the type IGF receptor, which mediates most biological functions of IGF-I and IGF-II, is down-regulated during carcinogenesis (4). In benign prostatic hyperplasia (BPH), a non-cancerous abnormality of human prostate, there may be increased response of prostatic stromal cells to IGF-II (3). Additionally, recent epidemiological studies have shown an increased risk of developing prostatic carcinoma in adult males with high-normal serum concentrations of IGF-I (5). Despite extensive characterization of the IGFBPs in the human prostate, little is known about their roles in prostate physiology. IGFBP-2 through 6 are produced by both epithelial and stromal cells (6,7). Importantly, IGFBP-2 and -4 are known to increase during carcinogenesis of the prostate epithelium (6,7), while IGFBP-3 is a proteolytic substrate for prostate specific antigen (8,9). The characterization of the new IGFBP-rPs in the human prostate is, however, just emerging.

The IGFBP-rPs are cysteine-rich proteins involved in a diversity of biological functions including growth regulation. IGFBP-rP1, which has also been termed meningioma-associated cDNA (MAC25) (10), tumor-derived adhesion factor (TAF, recently renamed Angiomodulin) (11), prostacyclin-stimulating factor (PSF) (12), and T1A12 (13), was originally cloned from leptomeningial cells by subtractive hybridization and shown to be preferentially expressed in normal leptomeningial and mammary epithelial cells, compared to their counterpart tumor cells (10). Subsequently,

IGFBP-rP1 was shown to increase during senescence and in response to retinoic acid in normal mammary epithelial cells (14). Furthermore, loss of heterozygosity of the IGFBP-rP1 locus has been reported in 50% of the cancer breast tissues examined, suggesting a tumor-suppressing role for IGFBP-rP1 in breast cancer (13).

In the human prostate, we have also recently described preferential expression of IGFBP-rP1 in normal human prostate cells and tissues, compared to their malignant counterparts, and shown that this protein is up-regulated by TGF- $\beta$ 1 and atRA in prostate epithelial cells (15). Moreover, when the human IGFBP-rP1 cDNA was transfected into the tumorigenic M12 cells, significant growth inhibition was apparent, both *in vitro* and *in vivo* (16). Thus, IGFBP-rP1 appears to be a tumor suppressor gene, whose expression is abrogated in a number of cancer states, including meningiomas, breast and prostate cancer.

IGFBP-rP2 (also known as connective tissue growth factor/CTGF) was initially isolated from human umbilical endothelial cells and shown to be mitogenic and chemotactic for fibroblasts (17). IGFBP-rP2 belongs to the CCN (for CYR61, CTFG and NOV) family of cysteine-rich proteins involved in a diversity of cellular functions, such as adhesion, migration, mitogenesis, differentiation, survival and regulation of matrix gene expression (18). Additionally, this protein has been implicated in wound healing, angiogenesis, chondrogenesis, reproduction, tumorigenesis, fibrotic and vascular disorders (19,20).

Although the role of IGFBP-rP2 in tumorigenesis has not been clearly established yet, this protein has been detected in the stromal component of breast and pancreatic cancer and in the

endothelial component of non-malignant vascular tumors (21,22). Additionally, it has been shown that IGFBP-rP2 is up-regulated by TGF- $\beta$ 1 in the mammary cancer cell line Hs578T, suggesting that this protein is also secreted by the epithelial component of breast cancer (23). Furthermore, IGFBP-rP2 appears to induce apoptosis in the estrogen receptor-positive breast cancer cell line MCF-7 (24). Thus, IGFBP-rP2 is a new growth regulator involved in a wide variety of biological functions, including its emerging role in tumorigenesis. To date, however, no studies have been reported on the role of IGFBP-rP2 in prostate biology and/or carcinogenesis.

IGFBP-rP3 (also known as NOV, for nephroblastoma overexpressed) was first recognized as an aberrantly expressed gene in avian nephroblastoma and later shown to be also overexpressed in the human homologue Wilms tumor (25,26). In the latter, the concentrations of NOV mRNA were inversely correlated with those of the tumor suppressor gene *WT1*. These observations, as well as the demonstration that overexpression of an amino-terminal truncated fragment of NOV was sufficient to transform chicken embryo fibroblast, have suggested that *NOV* is a proto-oncogene (25). However, the same report also showed that overexpression of the full length NOV was inhibitory for these cells (25). Thus, the role of NOV as a growth regulator still remains to be elucidated. In the human prostate, it has been reported that NOV is differentially expressed in PC-3 cells, compared to other cancerous and normal cells in culture, but the significance of this finding is unknown (27).

We now report that prostate epithelial cells in culture express not only IGFBP-rP1 and 3, but also IGFBP-rP2, and that these proteins are responsive to growth regulators. Interestingly, IGFBP-rP1

and 2 also increase during senescence of normal prostate epithelial cells, thus supporting a growth-inhibitory role of these proteins in the prostate epithelium.

## **Material and Methods**

### *Materials*

Epidermal growth factor (EGF), dexamethasone, all-*trans*-retinoic acid, and the additive ITS (insulin, transferrin, selenium) were purchased from Sigma Chemical Co. (St. Louis, MO). PrEBM HPEC media was obtained from Clonetics (San Diego, CA). RPMI was obtained from Life Technologies (Grand Island, NY). IGF-I was a gift from Eli Lilly & Co. (Indianapolis, IN). TGF $\beta$ 1 was purchased from Austral Biologicals (San Ramon, CA). FBS was obtained from HyClone Laboratories, Inc. (Logan, UT). Nitrocellulose and electrophoresis reagents were purchased from Bio-Rad laboratories, Inc. (Hercules, CA); nylon membranes (Genescreen) were obtained from New England Nuclear (Boston, MA). Horseradish peroxidase-linked donkey anti-rabbit and sheep anti-mouse IgG antibodies and enhanced chemiluminescence detection reagents were purchased from Amersham (Arlington Heights, IL). HPEC cells were purchased from Clonetics (San Diego, CA). LNCaP, DU145 and PC-3 cells were obtained from American Type Culture Collection (Manassas, VA). P69 and M12 were kindly provided by Dr. Stephen R. Plymate (University of Washington). Polyclonal antibodies against IGFBP-rP1, IGFBP-rP2 and IGFBP-rP3 were generated in rabbits, as



previously described (23,28,29). Monoclonal IgG antibody against p16<sup>INK4a</sup> (13251A) was purchased from Pharmingen (San Diego, CA).

### *Cell culture*

Figure 1 summarizes the cell lines employed in these studies. HPEC cells were maintained in PrEBM media supplemented with PrEGM, that includes the following: bovine pituitary extract (BPE), insulin, hydrocortisone, GA-100, retinoic acid, transferrin, levothyroxine, epinephrine and human epidermal growth factor. HPEC were subcultured as recommended in the provider's protocol. When they reached 80% confluence, all growth factors were withdrawn, except for BPE, for 24 hours. Media were changed again to PrEBM plus BPE for an additional 48 hours, after which conditioned media, cell lysates and total cytoplasmic RNA was collected by standard protocols.

P69 and its M12 subline were grown in RPMI media supplemented with 10 ng/ml EGF, 0.1  $\mu$ mol/L dexamethasone, 5  $\mu$ g/mL insulin, 5  $\mu$ g/mL transferrin and 5 ng/mL selenium. LNCaP, DU145 and PC-3 cells were maintained in RPMI enriched with 10% FBS. All cultures used were mycoplasma-free, as determined by the Mycoplasma PCR Primer Set (Stratagene, La Jolla, CA) and were grown at 37 C under 5% CO<sub>2</sub>.

### *Growth factor treatment studies*

All cell lines were grown to 80% confluence in 60-mm tissue culture dishes and treated with various doses of TGF- $\beta$ 1 (0 - 5 ng/ml), atRA (0.001 - 1  $\mu$ mol/L) or IGF-I (0 - 100 ng/ml) under serum-

free conditions for 48 hours before collecting media, total cell lysates and total cytoplasmic RNA for Western immunoblots (see *Western immunoblot analyses*) and Northern blots (see *RNA analyses*) studies. All experiments were repeated at least twice.

#### *Western immunoblot analyses*

Conditioned media and total cell lysates using RIPA buffer [150 mM NaCl, 20 mM Hepes (pH 7.4), 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS and Mini EDTA-free protease inhibitors (Roche Molecular Biochemicals, Laval, Québec, Canada)] from both treated and untreated (control) cells were normalized for protein concentration, using the Bio-Rad DC (Bio-Rad Laboratories, Hercules, CA) protein assay. Equal amounts of total protein per sample were dissolved in non-denaturing SDS sample buffer [0.5 mol/L Tris (pH 6.8), 1% SDS, 10% glycerol and bromophenol blue] and boiled for 5 min. Samples were electrophoresed on 15% SDS-polyacrylamide gels, then electroblotted onto nitrocellulose and membranes blocked with 4% milk-TBS-T [Tris-buffered saline-Tween-20 (0.1%)] for 1 h at 22 C. Western blots were incubated with IGFBP-rP1, -rP2 or -rP3 antisera at a 1:3000 dilution and with p16<sup>INK4a</sup> IgG antibody at a dilution of 1:500 (1 µg/ml) in TBS-T overnight at 4 C. Blots were washed with TBS-T and then incubated for 1 h at 22 C with a 1:3000 dilution of horseradish peroxidase-linked anti-rabbit or anti-mouse IgG secondary antibodies. Proteins of interest were detected with ECL chemiluminescence reagents, according to the manufacturer's protocol.

### *RNA analyses*

Total cytoplasmic RNA was isolated from cells, by use of RNeasy (Qiagen, Inc., Chatsworth, CA). Twenty  $\mu\text{g}$  of each RNA preparation were electrophoresed on a 1.2% agarose-2.2 mol/L formaldehyde gel, transferred overnight onto a nylon membrane (GeneScreen, DuPont, Wilmington, DE) using 10 x SSC (standard saline citrate) as the transfer solution, and cross-linked to the membrane by UV irradiation in a Stratalinker 1800 (Stratagene, La Jolla, CA). The Northern blots were then probed with an EcoRI/Xho fragment of IGFBP-rP1 (30), or a BamHI/Xho fragment of IGFBP-rP2 (31), which were radiolabeled ( $1 \times 10^9$  dpm/ $\mu\text{g}$ ) with [ $\alpha$ - $^{32}\text{P}$ ]deoxy-CTP (New England Nuclear-DuPont; SA, 3000 Ci/mmol) using a random priming kit (Prime-a-Gene, Promega Corp., Madison, WI). Northern blots were hybridized overnight at 65 C in hybridization buffer (Rapid-Hyb, Amersham), according to the manufacturer's instructions. Blots were then washed for 15 min in 2 x SSC at 22 C, followed by two more stringent washes in 0.2 x SSC/0.1 % SDS at 65 C for 15 min. Blots were exposed to Kodak Biomax film (Eastman Kodak Co., Rochester, NY) for 12 to 48 h at - 70 C, using one intensifying screen. Membranes were then reprobed with 18S ribosomal RNA, which acted as a loading control for the RNA samples. An image analyzer (GS-700) equipped with Multi-Analyst version 1.0.2 Software (Bio-Rad Laboratories, Hercules, CA) was used to quantify the resulting bands.

### *RT-PCR*

RT-PCR was performed using 5' and 3' primers specific for IGFBP-rP3. One  $\mu$ g of total RNA from each cell line was reversed transcribed in a volume of 20  $\mu$ l, by use of Reverse Transcription System Kit (Promega Corp., Madison, WI) following the manufacturer's instructions. The reaction was performed at 42 C for 15 min, denatured at 99 C for 5 min and placed on ice. One  $\mu$ l of the first strand was then increased to a volume of 50  $\mu$ l by the addition of 50 pmols of 5' and 3' primers, 10 mM dNTPs, GC melt, reaction buffer and polymerase (Advantage GC cDNA PCR Kit, Clontech, Palo Alto, CA). Amplification of the cDNA was carried out with 25 cycles of denaturing at 94 C for 1 min, annealing at 55 C for 1 min, and extension at 72 C for 2 min. One negative and one positive control were included in all reactions.

## Results

### *Expression of IGFBP-rP1, 2 and 3 in human prostate epithelial cells*

In agreement with previous observations (15), IGFBP-rP1 mRNA was detected by Northern blot analysis in the P69/M12 lineage and in primary cultures of prostate epithelial cells, with a parallel detection of IGFBP-rP1 in conditioned media from these cultures (Fig.2). In the malignant LNCaP, DU145 and PC-3 cells, IGFBP-rP1 mRNA was undetectable by Northern blot and an immunoreactive band was only present in cell lysates but not in conditioned media from these cells.

IGFBP-rP2 mRNA was detectable in all but one cell line as a single 2.4 kb band, consistent with the molecular size observed in most studies (20). IGFBP-rP2 protein was present in conditioned

media and in cell lysates from these cultures, with relative concentrations that closely reflected those of their mRNA. In contrast to IGFBP-rP1, both normal and malignant prostate epithelial cells express similar amounts of IGFBP-rP2 (Fig. 2). IGFBP-rP3 message was evaluated by RT-PCR in only a limited number of cell lines, but was identifiable in P69, M12, LNCaP and PC-3 cells (data not shown). IGFBP-rP3 protein was undetectable in conditioned media from HPEC, compared to readily detectable levels in the immortalized P69 cell line and malignant M12, DU145 and PC-3 cells (Fig. 2).

#### *Expression of IGFBP-rP1 and 2 during senescence of HPEC*

HPEC cells have a limited life span, with only about 15 population doublings before they enter senescence and are unable to replicate, even in the presence of serum. Interestingly, both IGFBP-rP1 and 2 were up-regulated upon increasing passage of these cells, with concentrations of both mRNA and secreted protein more than a 5-fold higher at late passages compared to early passages (Fig. 3A and B).

In support of an apparent increase of these proteins during senescence, we also investigated the concentrations of cell-cycle inhibitors that are known to be modified during cellular senescence, such as p21<sup>WAF1</sup> and p16<sup>INK4a</sup> (32). In agreement with a previous report in primary cultures of prostate epithelial cells (33), p21<sup>WAF1</sup> was barely identifiable in our cell lysate preparations, either at early or at late passages, whereas a marked up-regulation of p16<sup>INK4a</sup> in senescing cells was evidenced (7-fold increase compared to early passages. Fig 3C), paralleling the increase in IGFBP-rP1 and -rP2.

*IGFBP-rP2 is responsive to growth regulators in human prostate epithelial cells*

In malignant mammary epithelial cells (Hs578T), IGFBP-rP2 is regulated by TGF- $\beta$  (23). To determine whether IGFBP-rP2 was similarly regulated in prostate epithelial cells, we investigated the effect of growth inhibitors (TGF- $\beta$  and atRA), and growth factors (IGF-I) on IGFBP-rP2 expression in these cells. IGFBP-rP2 was markedly responsive to all three growth-regulators in normal HPEC whereas only modest regulation by either IGF-I or TGF- $\beta$  was observed in the other cells lines, perhaps reflecting the loss of receptors for IGF-I and for TGF- $\beta$  with tumorigenesis.

In HPEC, TGF- $\beta$  caused a dose-dependent increase in the protein concentrations of secreted IGFBP-rP2 examined at 48 hours of incubation under serum-free conditions. A maximum response equivalent to a 6-fold increase over the non-treated cells was observed at a dose of TGF- $\beta$  of 5 ng/ml. Similarly, treatment with atRA at a dose of 1  $\mu$ mol/L caused an increase of IGFBP-rP2 protein of about 4-fold over basal levels (Fig. 4A).

Interestingly, HPEC were also responsive to treatment with IGF-I. Furthermore, this effect was dependent on the replicative state of these cells, as highly-proliferative cells showed minimal regulation, but senescing cells decreased their secreted levels of IGFBP-rP2 by 90% upon treatment with IGF-I at physiological doses (100 ng/ml) (Fig. 4B). Whether this regulation was observed at a transcriptional level or whether it also involved post-transcriptional or postranslational modifications was not investigated in the present study.

In PC-3 cells, the effect of TGF- $\beta$  on IGFBP-rP2 concentrations in conditioned media was also significant, with a 4-fold increase over non-stimulated values. Treatment with atRA, under the same experimental conditions, resulted in a 7-fold increase in IGFBP-rP2 concentrations (Fig 4A). In addition, we treated all of the other cell lines with the three above-mentioned growth-regulators, but only small responses were observed for TGF- $\beta$ , atRA and IGF-I in P69 cells and for atRA in M12 and DU145 cells (data not shown).

#### *IGFBP-rP3 is regulated in human prostate epithelial cells*

IGFBP-rP3 has been previously shown to be expressed in prostate epithelial cells, but no regulation has been described as yet in these cultures (27). To better understand the role of this protein in the human prostate, we also investigated the effects of TGF- $\beta$ , atRA and IGF-I on the expression of IGFBP-rP3 in vitro.

IGFBP-rP3 was not produced by normal prostate epithelial cells, nor was it induced by any of the above-mentioned growth-regulators in these cells. In the well-established DU145 and PC-3 cell lines, IGFBP-rP3 was readily detected in conditioned media under serum-free incubation, but no significant regulation was observed in these cells. Interestingly, in both P69 and M12 cells, IGFBP-rP3 was markedly down-regulated by the growth inhibitor atRA (4- and 2-fold, respectively), consistent with its role as a proto-oncogene (Fig. 5).

## Discussion

Recent studies indicate that both IGFBP-rP1 and IGFBP-rP3 are expressed by the prostate epithelium (15,27). We now report that prostate cells in culture also synthesize another member of the IGFBP Superfamily: IGFBP-rP2. Although the biological roles of these three proteins in prostate have yet to be defined, they are likely to play a role in the regulation of proliferation of prostatic cells, since they are responsive to growth regulators and increase (IGFBP-rP1 and 2) during senescence of the normal prostate epithelium.

IGFBP-rP1 (also known as Mac25 (10), TAF/Andiomodulin (11,34), PSF (12) and T1A12 (13)) has been shown to be differentially expressed in normal meningeal, breast and prostate cells, compared to their malignant counterparts (10,14,15). In the latter report, it was suggested that IGFBP-rP1 might have an antiproliferative effect in the prostate, as it was also up-regulated by the growth inhibitory TGF- $\beta$ 1 and atRA in prostate cells (15). Indeed, a more recent description indicates a possible role of IGFBP-rP1 as a tumor suppressor gene for prostate cancer, since overexpression of its gene caused an antiproliferative effect *in vitro* and *in vivo* in malignant M12 cells, which were shown to become more sensitive to pro-apoptotic agents (16). More difficult to reconcile, however, is a recent report indicating an up-regulation of IGFBP-rP1 during prostate carcinogenesis (35). Since the data by Degeorges *et al.* are based upon unconfirmed immunohistochemical studies, it is possible that the



differences found are attributable to alternative properties or specificity of their antibody; alternatively, different sources of prostatic epithelial cells may vary in these properties.

Further evidence supporting the role of IGFBP-rP1 as a tumor suppressor gene relates to its up-regulation during senescence of normal epithelial cells. Swisshelm *et al.* have described an enhanced expression of IGFBP-rP1 (3 to 4-fold increase at late passages compared to early passages) in senescent human mammary epithelial cells (HMEC), indicating a possible involvement of this protein in the cell-cycle mechanisms leading to cellular senescence (14). HPEC, similarly to HMEC, have a limited life span, undergoing senescence after 15 population doublings. Although we have not ascertained the precise replication times of HPEC in culture, it was evident that at late passages ( $\geq 8^{\text{th}}$ ) cells replicated at a much lower rate, requiring additional time to reach the desired confluence. Consistent with this was the marked up-regulation of the cell-cycle inhibitor p16<sup>INK4a</sup> at late passages of HPEC, occurrence that has been recently reported in these cells (33). Our results are, thus, in agreement with observations in human mammary cells, in that the concentrations of both IGFBP-rP1 mRNA and protein were markedly enhanced during senescence of normal prostate epithelial cells. Thus, although the role of IGFBP-rP1 in the human prostate has as yet to be clarified, our results support the hypothesis that IGFBP-rP1 is a tumor suppressor gene and/or senescence factor.

IGFBP-rP2 (also known as CTGF (17)) exerts growth-promoting properties in both fibroblast and endothelial cells, where it also enhances attachment, chemotaxis and synthesis of extracellular matrix (reviewed in (20)). In addition, it has been shown to participate in wound healing (36),

reproduction (37), fibrotic and vascular disorders (38), and recently also angiogenesis (39) and chondrogenesis (40).

Recent studies also support a role of IGFBP-rP2 in tumorigenesis. IGFBP-rP2 is expressed by a chondrosarcoma and a fibrocarcoma cell lines in vitro (20,41) and has been found in the stromal component of breast and pancreatic cancer and desmoplastic melanomas (21,22,42). Despite the lack of detection of IGFBP-rP2 in the epithelial component of breast cancer in one report (21), it has been shown that IGFBP-rP2 is up-regulated by TGF- $\beta$ 1 in the breast cancer cell line Hs578T (23) and that IGFBP-rP2 induces apoptosis in the estrogen receptor-positive breast cancer cell line MCF-7 (24). All of these lines of evidence suggest that IGFBP-rP2 plays a role modifying the growth of stroma in desmoplastic tumors and in regulating the growth of breast cancer cells.

In this light, our results indicate that IGFBP-rP2 may also regulate the growth of normal and prostate cancer cells. In normal HPEC, IGFBP-rP2 is markedly enhanced during senescence, in a similar fashion to IGFBP-rP1. Additionally, IGFBP-rP2 is noticeably up-regulated by growth inhibitory factors, such as TGF- $\beta$  and atRA in normal and malignant cells, and down-regulated by growth-promoting factors, such as IGF-I in HPEC. These observations suggest that IGFBP-rP2 may act as a growth inhibitor, inducing senescence in normal cells and mediating the effects of other growth inhibitors on cell-cycle progression and/or apoptosis in the normal and malignant prostate. Indeed, IGFBP-rP2 has been shown to be a modulator of cell cycle progression in cAMP-arrested fibroblasts (43,44).

IGFBP-rP3 (also known as NOV (25)) is overexpressed in Wilms tumor, showing an inverse correlation with the concentrations of the tumor-suppressor gene *WT1*(26), which suggests a potential role of this protein as an proto-oncogene. Supporting this hypothesis is the observation that overexpression of the amino-terminal truncated NOV molecule was also able to transform chicken embryo fibroblasts (25). More recently, NOV has also shown to induce proliferation of mouse fibroblasts *in vitro* and to enhance phosphorylation of a 221 kDa protein, suggesting growth-stimulatory properties of this protein through activation of a still unidentified phosphorylated molecule (45).

In the prostate, data on NOV expression are only modest, with a report showing that NOV mRNA, by RT-PCR, is preferentially expressed in PC-3, compared to other normal and malignant prostatic epithelial cells (27). Our results are in agreement with the report by Tatoud *et al*, in that IGFBP-rP3 was highly expressed in PC-3 cells. However, by RT-PCR, we were also able to detect IGFBP-rP3 in the immortalized P69 cells and in the malignant M12 and LNCaP lines. Moreover, IGFBP-rP3 protein was also detected in conditioned media from all of these cells (except LNCaP) and in the malignant DU145 cells, but not in normal HPEC cells. Additionally, IGFBP-rP3 expression was suppressed by the growth-inhibitors TGF- $\beta$  and atRA in P69 and M12 cells. Thus, IGFBP-rP3 expression in prostatic cells is consistent with its potential role as a proto-oncogene and/or growth factor, given its lack of detection in normal cells, the induction of its gene during transformation and tumorigenesis, and its suppression with growth inhibitory factors.

In summary, we report on the expression and regulation of two additional IGFBP-rPs in prostate cells: IGFBP-rP2 and 3, with results that support the hypothesis that these proteins, like IGFBP-rP1, are involved in the regulation of prostatic cell growth. IGFBP-rP2 may play a role as a growth inhibitor, since its expression is: 1) enhanced during senescence of normal prostate epithelial cells (in a similar fashion to IGFBP-rP1); 2) increased by growth inhibitory factors (TGF- $\beta$  and atRA); and 3) decreased by IGF-I. Conversely, IGFBP-rP3 may act as a growth factor for prostate cells, given its preferential expression in malignant cells and its down-regulation by atRA.

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## FIGURE LEGENDS

**Figure 1. Prostate cancer epithelial cells studied.** Primary cultures of normal human prostate epithelial cells (HPEC) are mainly basal epithelial cells. After transformation with the SV40-T antigen, the cell line P69SV40T (P69) was obtained with low tumorigenic potential. In contrast, M12 cells, originated after several passages of P69 cells in athymic mice, are highly tumorigenic and metastatic. The well-established androgen-dependent (LNCaP) and androgen-independent (DU145 and PC-3) cells lines, derived from clinical samples, were also studied.

**Figure 2. Expression of IGFBP-rP1, 2 and 3 in human prostate epithelial cells. A .** Western immunoblot studies of conditioned media (C) and total cell lysates (L) from prostate cell lines using polyclonal anti-IGFBP-rP1, 2 and 3 antibodies. Ten  $\mu$ g of total protein was loaded per lane. HPEC<sub>4</sub>: 4<sup>th</sup> passage (highly-replicative cells). HPEC<sub>9</sub>: 9<sup>th</sup> passage (slowly-dividing cells). IGFBP-rP1 is highly expressed in normal prostate epithelial cells with low or undetectable concentrations in the malignant M12, LNCaP, DU145 and PC-3 (To avoid over-saturation of the film, only 5  $\mu$ g of total protein for HPEC conditioned media was loaded per lane on the IGFBP-rP1 immunoblot). Conversely, IGFBP-rP3 is undetectable in HPEC but expressed in the immortalized P69 and malignant M12, DU145 and PC-3 cells. IGFBP-rP2 was expressed in both normal and malignant cells. **B.** Northern blot analyses of prostate cells using [32P] radiolabelled IGFBP-rP1 and 2 probes. Twenty  $\mu$ g of total RNA was loaded per lane. Concentrations of 18S ribosomal RNA are shown as an internal control for loading.

**Figure 3. Expression of IGFBP-rP1 and 2 during senescence of HPEC .** **A.** Western immunoblot studies of conditioned media by HPEC using polyclonal anti-IGFBP-rP1 and 2 antibodies. Ten  $\mu\text{g}$  of total protein was loaded per lane. At low passages ( $\leq 4^{\text{th}}$ ) cells are highly replicative. At late passages ( $\geq 9^{\text{th}}$ ) cells start to senesce and show a marked increase in their replication time. Both IGFBP-rP1 and 2 concentrations in conditioned media increased  $\approx 6$ -fold at late, low-replicative passages compared to early ones. **B.** Northern blot analyses of same serial passages of HPEC using  $[^{32}\text{P}]$  radiolabelled IGFBP-rP1 and 2 probes. Twenty  $\mu\text{g}$  of total RNA was loaded per lane. Concentrations of 18S ribosomal RNA are shown as an internal control for loading. **C.** Western immunoblot studies of total cell lysates from HPEC using monoclonal anti-p16<sup>INK4a</sup> antibody. Ten  $\mu\text{g}$  of total protein was loaded per lane. Note a marked up-regulation ( $\approx 6$ -fold) of p16<sup>INK4a</sup> concentrations at late, low-replicative passages compared to early ones, as part of the senescence process of normal cells.

**Figure 4. IGFBP-rP2 is responsive to growth regulators in human prostate epithelial cells .** **A.**

Up-regulation of IGFBP-rP2 by the growth inhibitors TGF- $\beta$  and atRA. Representative Western immunoblot of conditioned media by HPEC and PC-3 cells using polyclonal anti-IGFBP-rP2 antibody. Cells were treated with increasing concentrations of TGF- $\beta$ 1 and atRA for 48 hours in serum-free conditions. Densitometric analysis of IGFBP-rP2 signal is also shown graphically (Mean  $\pm$  SE of three independent experiments). A 4-fold increase or greater was seen with both TGF- $\beta$ 1 and atRA in both

HPEC and PC-3 cells. **B.** Down-regulation by the growth factor IGF-I. Representative Western immunoblot of conditioned media by HPEC using polyclonal anti-IGFBP-rP2 antibody. Three different passages are shown in this blot: early (4<sup>th</sup>), intermediate (7<sup>th</sup>) and late (9<sup>th</sup>). Cells were treated with increasing concentrations of IGF-I for 48 hours in serum-free conditions. Densitometric analysis of IGFBP-rP2 signal is also shown graphically. A 90% decrease in IGFBP-rP2 was seen at late passages of HPEC treated with IGF-I.

**Figure 5. IGFBP-rP3 is regulated in human prostate epithelial cells:** Down-regulation of IGFBP-rP3 by the growth inhibitor atRA. Representative Western immunoblots of conditioned media by P69 and M12 cells using polyclonal anti-IGFBP-rP3 antibody. Cells were treated with increasing concentrations of atRA for 48 hours in serum-free conditions. Densitometric analysis of IGFBP-rP3 signal is also shown graphically (Mean  $\pm$  SE of three independent experiments). In both P69 and M12 cells atRA caused a 4-fold and a 2-fold decrease, respectively, in the concentrations of IGFBP-rP3 in conditioned media.

Figure 1

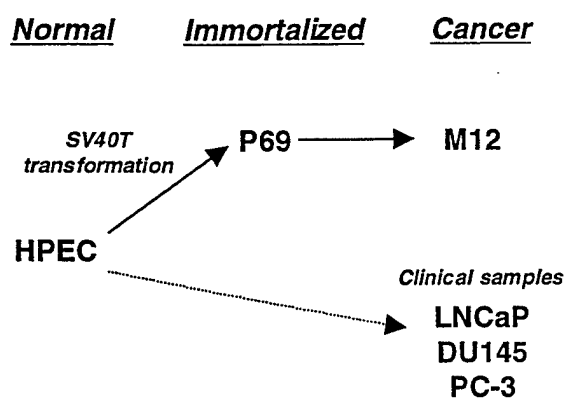


Figure 2

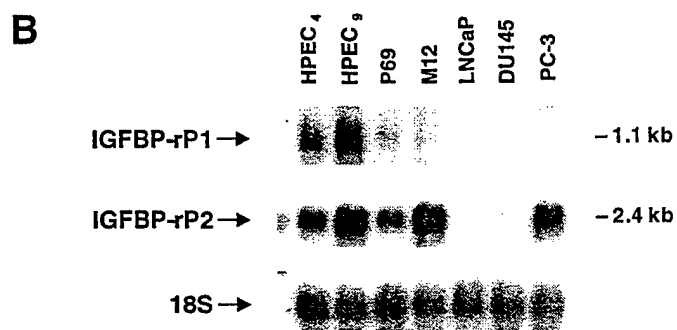
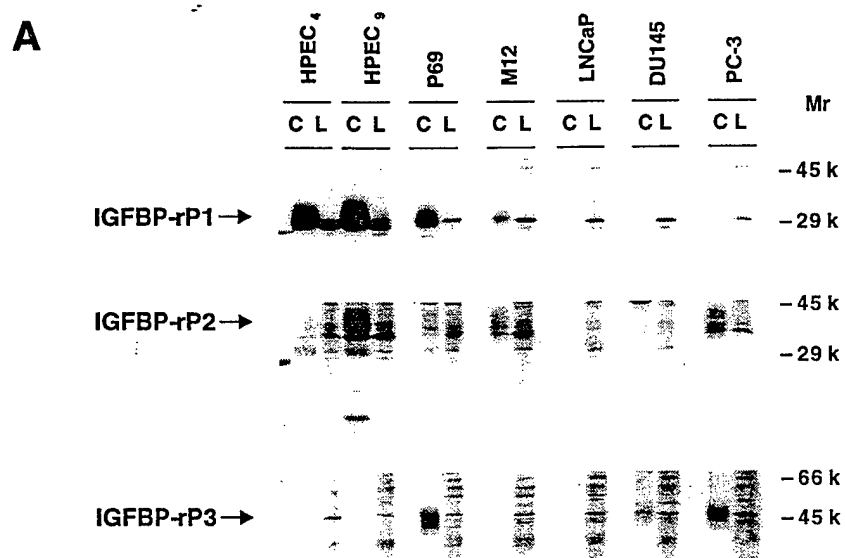




Figure 3

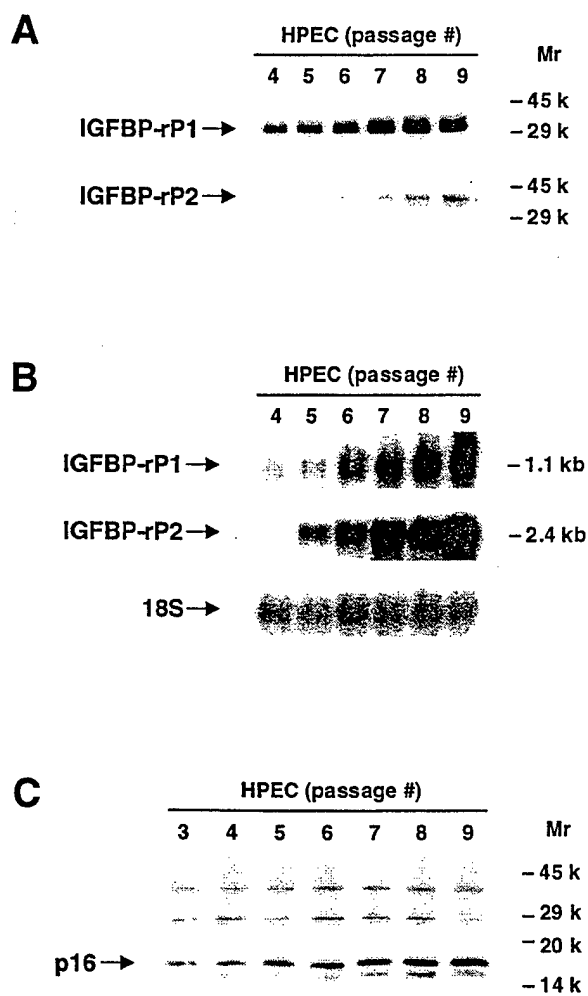


Figure 4

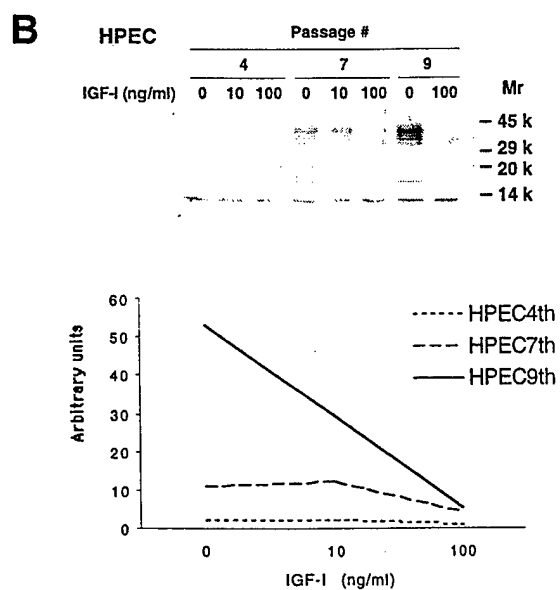
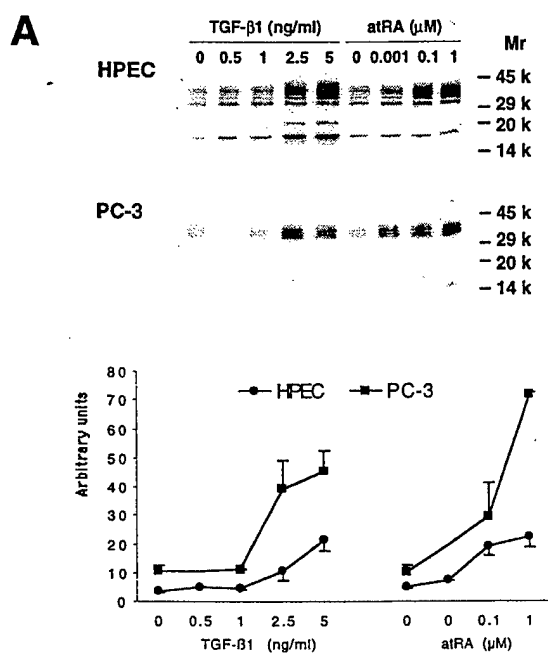
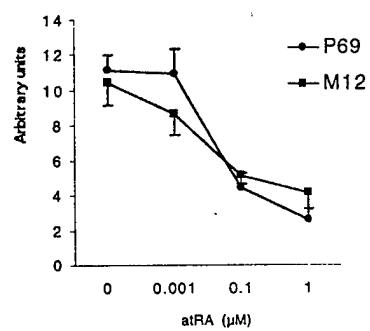
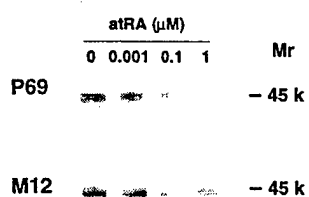
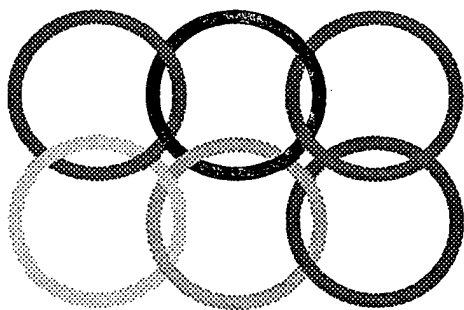


Figure 5



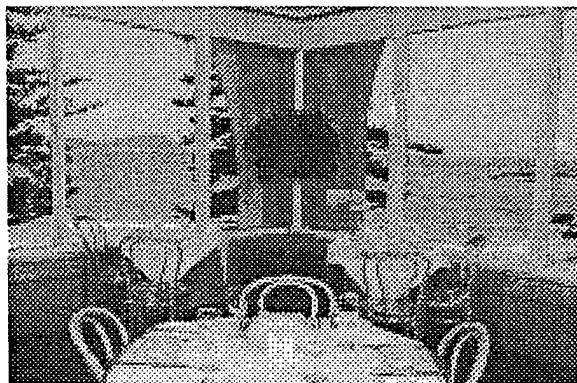


# IGFBP2000

4th International Workshop  
on IGF Binding Proteins  
October 26-29, 2000  
Terrigal, NSW, Australia

A SATELLITE MEETING TO ICE2000

On behalf of the International Society for Insulin-like Growth Factor Research, we invite you to attend the 4th International Workshop on IGF Binding Proteins, to be held at Terrigal, NSW, a beachside resort about 90 minutes drive north of Sydney.



The Workshop will open with registration, a welcome reception and dinner on Thursday October 26, 2000, with scientific sessions all day Friday and Saturday, and Sunday morning.

On Sunday afternoon, transport will be provided to the 11th International Congress of Endocrinology, Darling Harbour, Sydney, which opens on Sunday evening, with scientific sessions starting Monday October 30.

Like the previous IGFBP meetings held in Vancouver, Canada (1989), Opio, France (1992), and Tübingen, Germany (1995), the 4th International Workshop will feature invited talks by leading researchers, selected by an international Scientific Committee, as well as submitted contributions from delegates. These will be presented as posters apart from 20 abstracts selected by the committee for oral presentation. Special program features include 10 oral sessions, each beginning with an overview and discussion session led by an expert in the field, 17 talks from invited speakers on their current research, and 4 poster sessions.

We look forward to welcoming you in Terrigal in October 2000.

Janet Martin  
Rob Baxter  
Local Organising Committee

## IGFBP2000 Program

	THURSDAY 26 October	FRIDAY 27 October	SATURDAY 28 October	SUNDAY 29 October
8.30-10.15		IGFBP evolution and structure	IGFBP modulation of IGF action	IGFBPs <i>in vivo</i>
10.15-10.45		morning tea	morning tea	morning tea
10.45-12.30		IGFBP knockouts and transgenes	IGF-independent actions of IGFBPs	IGFBPs in the clinic
12.30-14.15		lunch and posters	lunch and posters	lunch and transfer to ICE2000
14.15-16.00		postranslational modification of IGFBPs	nuclear translocation of IGFBPs, and interacting proteins	
16.00-16.30		afternoon tea	afternoon tea	
16.30-18.00	registration	gene regulation of IGFBPs	IGFBP related proteins	
18.00-19.00	welcome reception	posters/social	posters/social	

## REGISTRATION AND ACCOMMODATION

The Workshop will be held in the Holiday Inn Resort, Terrigal, NSW, and has been planned as an all-inclusive 3-day package with delegates staying at the conference venue.

Note that attendance at the meeting is limited to 200 delegates. Registrations will be accepted on a first come, first served basis, with the following provision. Registrants are strongly encouraged to book the accommodation package offered together with registration. Due to the hotel's requirements that we occupy at least 160 rooms, we cannot guarantee that registration for the meeting can be accepted from anyone who does not also book the accommodation package.

When the registration limit has been reached, we will return registration forms and payments without processing them.

### Registration as a delegate

 entitles you to:

- attendance at welcome reception on October 26
- attendance at all sessions, including abstract book etc.
- morning and afternoon teas
- dinners on October 26, 27 and 28
- transport from Terrigal to the ICE2000 meeting on October 29

### Registration as an accompanying person

 entitles you to

- all of the above except attendance at sessions and abstract book etc.

Accommodation rates include your room for the nights of October 26, 27 and 28, and breakfasts and lunches on October 27, 28 and 29.

Details of transport from Sydney Airport to the conference venue at Terrigal, NSW will be included with the confirmation of registration.

#### SCIENTIFIC COMMITTEE

Janet Martin, Chair (*Australia*)

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Michel Binoux (*France*)

Kerstin Brismar (*Sweden*)

David Clemmons (*USA*)

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Dear Dr Devi

I am pleased to inform you that your abstract titled  
OVEREXPRESSION OF IGFBP-3 INDUCES EARLY APOPTOSIS AND CELL GROWTH  
INHIBITION IN MALIGNANT PROSTATE CELLS  
has been selected for poster presentation at IGFBP2000. I will forward  
further details about the poster boards which will be available, and  
presentation times, shortly.

It is also my pleasure, on behalf of the scientific and local  
organizing  
committees of IGFBP2000, to be able to offer you a travel award of  
AUD\$1000. This will be paid to you at the conference in October. We  
would  
also like to offer you complimentary registration for the conference.  
If

you have sent your registration payment for IGFBP2000, you will be  
reimbursed for this at the meeting. If you have not already arranged  
for  
registration payment, please indicate on your completed registration  
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that you are not required to pay for registration. This form should  
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the office of IGFBP2000 by July 31st (fax to 61-2-9926-8484).  
Our arrangements with the meeting sponsor and Holiday Inn Terrigal, the  
conference venue, require that accommodation payments be made in  
advance of

the conference. Delegate numbers are strictly limited, so in order to  
confirm your attendance at the meeting we would appreciate receiving  
your

accommodation payment by July 31st, or as soon as possible thereafter.  
Further information about the conference, including details of  
transport to

Terrigal, will be sent to you with confirmation of your registration  
and  
accommodation payments.

Please confirm receipt of this email indicating your acceptance of the  
offer of a travel grant and registration. You will also need to forward  
a

letter from your supervisor or head of department confirming your  
student/trainee/fellow status in order to be eligible for this award.  
Once

again, congratulations, and we look forward to seeing you at IGFBP2000  
in  
October.

Mail

Best wishes  
Janet Martin  
on behalf of the scientific and Local Organizing Committees, IGFBP2000

IGFBP2000  
c/- Dr Janet I Martin  
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## **OVEREXPRESSION OF IGFBP-3 INDUCES EARLY APOPTOSIS AND CELL GROWTH INHIBITION IN MALIGNANT PROSTATE CELLS**

Devi, GR\*<sup>1</sup>, Sprenger, CC<sup>2</sup>, Graham DL<sup>1</sup>, Plymate, SR<sup>2</sup> and Rosenfeld, RG<sup>1</sup>

<sup>1</sup>Oregon Health Sciences University, Portland, OR 97021, USA; <sup>2</sup>University of Washington, Tacoma, WA 98493, USA.

Insulin-like growth factor binding protein (IGFBP-3) is secreted by prostate cell cultures. However, the IGFBP-3 levels are significantly reduced in malignant prostate epithelial cells (PEC) and in serum of patients with prostate cancer. Exogenous IGFBP-3 has been shown to block IGF action, inhibiting cell growth *in vitro*. The present study was carried out to evaluate the role of endogenous IGFBP-3 on prostate cell growth. M12 cells, a tumorigenic and metastatic PEC, was stably transfected with human IGFBP-3 cDNA (M12-BP-3) or with the vector pCDNA3 as control (M12-pCD). M12-BP-3 cells secreted IGFBP-3, whereas it was undetectable in the control cells. Growth of M12-BP-3 cells was significantly slower and growth arrest occurred at a cell density that was 3 -fold lower than the control cells. There was a marked alteration in the M12-BP-3 cell morphology from the characteristic cuboidal shape associated with epithelial cells to elongated with increased cytoplasmic/nuclear ratios. Co-culture experiments in which the control M12 and M12-pCD cells were grown in conditioned media secreted by M12-BP-3 cells also showed altered morphology and increased number of detached cells within 12-24 h of treatment, which was reversed on removal of the media containing BP-3. A neuroendocrine marker, NSE, which is highly expressed in malignant prostate cells, was decreased in M12-BP-3 and in the control cells growing in BP-3 conditioned media. Altered mitochondrial membrane potential, an early apoptotic event, and an increased PARP cleavage in response to 6-hydroxyurea, was observed in M12-BP-3 cells. Flow cytometric analysis of the adherent and detached cells revealed that within 24 h, 20-30% of the M12-BP-3 cells were in pre-G1 peak, a growth characteristic of apoptotic cells, compared to 2% in the case of M12-pCD. These data indicate that endogenous IGFBP-3 causes significant delay in the growth of malignant PEC and enhances the sensitivity of these cells to apoptosis.

GRD\* supported by US Army Grant DAMD17-99-1-9522



predicted) who participated in an oral corticosteroid sparing trial of inhaled fluticasone propionate (FP MDI). Subjects were randomized to receive FP 880mcg BID, FP 440mcg BID in HFA or CFC propellant or Placebo (P) in HFA propellant BID for 16-weeks. Prednisone (pred) dose allowed at entry was 5mg-40mg QD or 10mg-80mg QOD. Following a 2-week stabilization period in which pred dose was held constant or increased, pulmonary function, pred use, and asthma symptoms were assessed weekly to determine their ability to reduce their pred dose. Subjects unable to meet pred reduction criteria held their dose constant or increased based upon their pulmonary function. Effects on HPA-axis function were assessed via short cosyntropin infusion test. Abnormal response was defined as a post-stimulation rise in cortisol <7mcg/dl and a post-stimulation peak cortisol <18mcg/dl. Results at screening and Week 16 are presented below:

	Placebo HFA	FP 440 CFC	FP 880 CFC	FP 440 HFA	FP 880 HFA
Screening, n	32	36	31	32	30
Post-Stimulation Rise in Cortisol 7mcg/dl and post-stimulation peak cortisol 18mcg/dl	14 (44%)	20 (56%)	14 (45%)	19 (59%)	16 (53%)
Week 16, n	11	31	26	26	19
Post-stimulation rise in cortisol 7mcg/dl and post-stimulation peak cortisol 18mcg/dl	6 (55%)	10 (32%)	9 (35%)	9 (35%)	9 (47%)

HPA-Axis function improved in the FP treatment group and deteriorated in the placebo group. A significantly greater proportion of patients randomized to FP completed the trial compared to placebo. Total elimination or reduction of pred dose by at least half was achieved by 88%-94% of subjects in the FP groups compared to 41% of subjects in the P group. Adverse events reported were expected given the demographics and comorbidities of this patient population. In conclusion, FP MDI shows improvement in HPA function in this study and offers the possibility of withdrawal of oral prednisone therapy plus an excellent benefit risk profile in this population of asthmatic patients.

## Basic Science: IGFs

### 904\* IGF Signaling and Binding Proteins Oral Session, Thursday, 6/22, 1:00 PM, Room 701

**Insulin Resistance in Mice Deficient in Insulin-Like Growth Factor.** Shoshana Yakar, Li Lin, Ana M Fernandez, Yiping Wu, Derek LeRoith, <sup>1</sup>Clinical Endocrinology Branch, National Institute of Health, Bethesda, MD

Insulin and insulin-like growth factor-I (IGF-I) mediate a variety of signals involved in mammalian development and metabolism. The most prominent function of IGF-I is regulation of body growth and development whereas the major function of insulin is the maintenance of euglycemia that is achieved by stimulation of glucose uptake in muscle. To study the effects of IGF-I deficiency we utilized liver-specific IGF-I-deficient mice which show a significant reduction (~75%) in circulating levels of IGF-I and increased levels of growth hormone (GH). Serum insulin levels in the liver IGF-I-deficient mice were 4 fold greater than wild-type littermates (0.6 ng/ml versus 2.2 ng/ml). Glucose tolerance tests were similar between the IGF-I-deficient mice and their wild-type littermates. On the other hand, in the IGF-I-deficient mice, serum glucose levels failed to fall following insulin injections, whereas wild-type littermates showed a significant fall of 50% in serum glucose levels. The serum glucose fall in response to IGF-I was similar in both groups of animals. Thus the high levels of circulating insulin in the IGF-I-deficient mice explain the maintenance of normoglycemia in the face of insulin resistance. Insulin-induced receptor autophosphorylation and phosphorylation of IRS-1 were markedly inhibited in muscle, while normal in liver in IGF-I-deficient mice. IGF-I-induced autophosphorylation of its cognate receptor and IRS-1 was normal in these mice. Thus the insulin resistance seen in these liver IGF-I-deficient mice is not a result of the muscle insulin receptor. Since skeletal muscle is responsible for 80% of stimulated glucose transport, circulating IGF-I levels may serve to fine tune insulin sensitivity.

### IGF Signaling and Binding Proteins Oral Session, Thursday, 6/22, 1:00 PM, Room 701

**Levels of Insulin-Like Growth Factor Binding Proteins Are Altered in Liver-Specific Insulin Receptor Knockout Mice.** M. Dodson, Michael C. Ronald, Kahn, <sup>1</sup>Genetics Ctr and Dept of Medicine, Harvard Medical School, Boston, MA. Liver-specific insulin receptor knockout (LIRKO) mice were generated using the Cre-loxP system. At 2 months of age, LIRKO mice were severely hyperglycemic in the fed state and mildly hyperglycemic in the fasted state. Serum insulin levels in the same mice were 15-20 fold elevated in both the fasting and fed states as compared to controls. This is due to both a significant increase in islet mass and a decrease in insulin clearance by the liver. Intraperitoneal glucose and insulin tolerance tests at 2 months of age demonstrated that LIRKO mice are severely glucose

intolerant and completely resistant to the blood glucose lowering effects of insulin. Furthermore, LIRKO mice displayed mild growth retardation around the time of weaning (post-natal day 18) that persisted to varying degrees throughout life. The biological effects of growth hormone (GH) to promote tissue growth are mediated by insulin-like growth factor-1 (IGF-1), which is primarily produced by the liver. In the present study, we have assessed whether defects in the GH/IGF-1 axis are responsible for the growth defects in LIRKO mice. Interestingly, total IGF-1 levels in serum were elevated by 2-fold in LIRKO mice suggesting the possibility of IGF-1 resistance in the LIRKO mice. In agreement with this notion, sensitivity to IGF-1 as measured by glucose lowering to an intraperitoneal injection of IGF-1 was severely blunted in LIRKO mice. We reasoned that the IGF-1 resistance could be due to decreased IGF-1 receptor signaling in target tissues or to reduced bioavailability of circulating IGF-1 resulting from an alteration in circulating IGF binding proteins (IGFBPs). Western ligand blotting of serum from both fasted and fed LIRKO mice demonstrated elevations in the ~29-32 kDa IGFBPs, a virtual absence of the ~35-40 kDa IGFBP-3, and no change in the 24 kDa IGFBP-4. Northern blotting of liver RNA revealed that two of the ~29-32 kDa IGFBPs, IGFBP-1 and -2, were overexpressed by ~20-fold in LIRKO mice compared to controls. IGFBP-3 transcript levels in liver were not different in LIRKO mice as compared to controls indicating that the decrease in circulating IGFBP-3 occurs at a post-transcriptional step, such as proteolysis. These results further the notions developed in other studies that insulin inhibits IGFBP-1 gene transcription, decreases IGFBP-2 mRNA half-life and may affect IGFBP-3 gene expression in non-parenchymal cells by interaction with parenchymal cells. Our results support that insulin has direct effects on hepatocyte-specific expression of IGFBP-1 and -2, and further suggest that the effects on IGFBP-3 expression in non-parenchymal cells is mediated by an insulin-stimulated factor that is secreted from hepatocytes.

### 906\* IGF Signaling and Binding Proteins Oral Session, Thursday, 6/22, 1:00 PM, Room 701

**Effect of IGFBP-3 on IGF- and IGF-Analog-Induced IGFR Signaling.** Gayathri R Devi, Youngman Oh, Ron G Rosenfeld, <sup>1</sup>Pediatrics, Oregon Health Sciences University, Portland, OR

IGFBP-3 binds IGF-I and II with high affinity, at least an order of magnitude higher than the affinity of the IGFs for the IGFR. It has been hypothesized that IGFBP-3 might inhibit IGF binding to the IGFR via a mechanism independent of its ability to sequester IGFs in the extracellular environment. This is based on earlier studies that showed that IGFBP-3 was as potent as IGF-I in "displacing" des(1-3)IGF-I (which, depending on the type of *in vitro* binding assay, has been reported to have 25-100 fold lower binding affinity for IGFBP-3) from its binding to the cell surface, and in dissociating IGFR bound ligand. In the present study, we have extensively examined the ability of IGFBP-3 to modulate the initial event in IGF signaling pathway, i.e., IGFR autophosphorylation induced by IGF-I, -II, insulin and IGF-analogs. IGFBP-3 and the peptides were preincubated at 4°C and then added to NIH/3T3 cells overexpressing IGFR cells for 5 min, which revealed that IGFBP-3 inhibited IGF-I, -II, des(1-3)IGF-I and long R3-IGF-I-induced IGFR autophosphorylation in a dose-dependent manner (IC<sub>50</sub> range 5-7 nM). In order to determine whether this effect was specific to IGFBP-3, the effect of IGFBP-2 on receptor autophosphorylation was carried out under similar conditions. Results showed that IGFBP-2 also dose-dependently inhibited the signaling event induced by IGF-I, at similar concentrations as with IGFBP-3. However, the analog-induced receptor autophosphorylation was inhibited by IGFBP-2 at 2-4 fold higher concentrations than IGFBP-3. This correlates well with the observations from previous studies that IGFBP-2 has 2-3 fold lower affinity than IGFBP-3 for the IGF-I analogs. Further, insulin-induced IGFR and insulin-receptor autophosphorylation were not inhibited, even at higher concentrations of IGFBP-3. IGFR-autophosphorylation induced by QAYL-IGF-II (analog of IGF-II which has very low affinity for IGFBP-3) was also not inhibited by IGFBP-3. In addition, preincubation of cells with IGFBP-3, followed by washing and IGF-I treatment caused no inhibition of IGF-induced autophosphorylation, suggesting that IGFBP-3 requires binding to IGF-I to suppress the IGF-induced IGFR signaling. Cross-linking experiments using <sup>125</sup>I-IGFBP-3 indicated that there is no direct interaction of IGFBP-3 with the IGFR. Further, *in vitro* solution binding assays to characterize the affinity of IGFBP-3 for the analogs revealed that des(1-3)IGF-I and R3-IGF-I had only 25-50 fold lower affinity compared to IGF-I, which correlates well with the *in vivo* effects. Based on this detailed *in vitro* and *in vivo* characterization of the IGF analogs, studies involving use of analogs to look at IGF-independent actions of IGFBP-3 should be interpreted with caution. This study clearly demonstrates that the effect on the initial step of IGFR signaling by IGFBP-3 is purely due to its ability to sequester IGF as well as IGF analogs in the extracellular milieu and not due to any direct interaction of IGFBP-3 with the IGFR or a mechanism independent of its ability to bind IGFs. These studies do not preclude, however, the possibility that IGFBP-3 might have IGF-independent effects on other distal IGFR signaling events. Supported by NIH grant R01 DK51513 (RGR) and US Army Grant DAMD17-99-1-9522 (GRD).

P2-582

**CHARACTERIZATION OF IGF AND INSULIN LIGAND BINDING TO THE AMINO- AND CARBOXYL-TERMINAL IGFBP-3 PROTEOLYTIC FRAGMENTS.**  
Gayathri R. Devi,\* Doo-Hyun Yang, Ron G. Rosenfeld, Youngman Oh. Pediatrics, Oregon Health Sciences University, Portland, OR

Insulin-like growth factor binding protein-3 (IGFBP-3), the predominant serum IGF carrier protein, regulates cell growth by modulating the biological actions of IGF-I and -II, which are fundamental mitogenic and differentiative peptides. In addition to the IGF-dependent actions, IGFBP-3 has direct antiproliferative functions, via binding to specific cell surface receptors. IGFBP-3 may be post-translationally modified by IGFBP-3 protease(s), that degrade IGFBP-3 into smaller fragments. It has been hypothesized that proteolysis of IGFBP-3 modulates its biological activity. In the present study, we extensively examined the ability of the NH<sub>2</sub>-terminal (1-97), intermediate fragment (88-148) and COOH-terminal proteolytic fragments (98-264 and 184-264) to bind IGF and insulin and to modulate IGF-I-induced IGF-I receptor (IGFIR) autophosphorylation. Based on the ascertained and predicted recognition sites for known IGFBP-3 proteases, such as prostate specific antigen and plasmin, both intact IGFBP-3 and four fragments were generated in a baculovirus expression system and/or in *E. coli*. Each fragment was tagged with a FLAG epitope at the COOH-terminus. To determine whether proteolysis of IGFBP-3 affected affinity or specificity for IGF and insulin ligands, two approaches, western ligand blot and affinity cross-linking were carried out. The NH<sub>2</sub>- and COOH-terminal fragments specifically bound both IGF and insulin, whereas the 88-148 fragment failed to bind IGF, which previously has been identified to contain structural determinants for association with the putative IGFBP-3 receptor. The (1-97) NH<sub>2</sub>- and the (98-264) COOH-terminal proteolytic fragments had significantly reduced affinity for IGF than intact IGFBP-3. Interestingly, the 1-97 fragment had 2- to 3-fold lower affinity for IGF in comparison to the (98-264) IGFBP-3 fragment. However, both these fragments had higher affinity for insulin than did intact IGFBP-3. Further, the effect of IGFBP-3 on IGFIR signaling pathways was studied by testing the receptor autophosphorylation in IGFIR-overexpressing NIH/3T3 cells. IGFBP-3 showed a dose-dependent inhibition of IGF-I-induced autophosphorylation of the  $\beta$ -subunit of IGFIR. Preliminary results show that the (98-264) COOH-terminal fragment also inhibits IGFIR autophosphorylation, albeit at higher concentrations than intact IGFBP-3. This is the first study demonstrating the ability of the COOH-terminal region of IGFBP-3 to bind both IGF and insulin. However, high-affinity binding of IGFBP-3 to IGFs requires proper structural configuration involving both NH<sub>2</sub>- and COOH-terminal ligand binding domains and the proteolytic fragments generated by proteases, may not retain the ability to inhibit cell proliferation by modulating IGF binding to IGFIR. Alternatively, given the high-affinity of the proteolytic fragments for insulin, they could modulate cell growth through an IGF-independent mechanism.

P2-583

**BIOSENSOR MEASUREMENT OF THE BINDING OF INSULIN-LIKE GROWTH FACTOR I AND II TO A N-TERMINAL RECOMBINANT INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 FRAGMENT.**

Peter Vorwerk,\*<sup>1</sup> Bianka Hohmann,<sup>1</sup> Youngman Oh,<sup>2</sup> Ron G. Rosenfeld,<sup>2</sup> Ronald M. Shymko.<sup>3</sup> <sup>1</sup>Pediatric Hematology & Oncology, Otto-von-Guericke-University, Magdeburg, Germany, <sup>2</sup>Pediatrics, Oregon Health Sciences University, Portland, OR, <sup>3</sup>Scientific Computing, Hagedorn Research Institute, Gentofte, Denmark

IGFBP-3 can be proteolyzed under various conditions. Initially described in human pregnancy serum, limited proteolysis of IGFBP-3 became subject of intensive research. Reports about limited proteolysis of IGFBP-3 and the physiological relevance of the resulting fragments have provided different results with respect to binding ability of these fragments to IGFs. Estimations of the binding affinity of IGFs to various smaller IGFBP-3 fragments by western ligand blot, affinity crosslinking or solution binding assay showed no or weak binding; however exact calculations of affinities from these methods are difficult. Aim of the present study was to investigate binding kinetics of IGF-I and -II to a recombinant expressed IGFBP-3 fragment using a BIAcore™-Biosensor (Pharmacia). Furthermore the effect of IGF-I biotinylation of the binding to IGFBP-3 and its fragments was determined. A N-terminal IGFBP-3 fragment (amino acids 1-97) was expressed in *E. coli* and purified from cell-lysate using a C-terminal fused FLAG epitope. IGF biotinylation was done as published by Fowlkes (Fowlkes et al. Endocrinology 1996; 137(12); 5751-5754). IGFBP-3 protein and fragments were immobilized covalently to a sensor chip in the BIAcore instrument, and IGF-I or IGF-II passed over the chip in a solution.  $K_d$  values from IGF-I, IGF-II and biotinylated IGF-I to IGFBP-3 and the IGFBP-3 fragment are shown in the table below. Our data suggest a 1000-fold lower binding affinity from IGFs to the N-terminal IGFBP-3 fragment in contrast to intact IGFBP-3. Biotinylated IGF-I has about 45 times less affinity for IGFBP-3 than IGF-I although the N-terminal fragment showed less significance between the two ligands.

peptide	IGFBP-3	IGFBP-31-97
IGF-I	$2.1 \times 10^{-10}$	$1.1 \times 10^{-7}$
IGF-II	$1.5 \times 10^{-10}$	$3.03 \times 10^{-8}$
IGF-I biotin.	$9.04 \times 10^{-9}$	$3.2 \times 10^{-7}$

P2-584

**LACTOFERRIN SPECIFICALLY BINDS TO IGFBP-3 TO CAUSE COMPETITIVE DISPLACEMENT OF IGF FROM IGF: IGFBP-3 COMPLEXES AND INDUCES INTERNALIZATION AND NUCLEAR LOCALIZATION OF LACTOFERRIN:IGFBP-3 COMPLEXES IN MAMMARY EPITHELIAL CELLS.**  
Craig R. Baumrucker,\*<sup>1</sup> C. Anne Gibson,<sup>1</sup> Yongfeng Shang,<sup>1</sup> Floyd L. Schanbacher,<sup>2</sup> Michael H. Green.<sup>1</sup> <sup>1</sup>Penn State University, <sup>2</sup>Ohio State University

We previously reported that rhIGFBP-3<sup>E.coli</sup> binds to a ~70 kDa mammary epithelial cell membrane protein that we hypothesized was an IGFBP-3 receptor or transport protein. By immunoprecipitation, we show that the ~70 kDa protein is bovine lactoferrin (bLf). Lf is a 77 kDa glycosylated and multifunctional iron-binding protein that is synthesized by many tissues, but is secreted by mammary epithelial cells in high concentrations during late prepartum periods, involution, and mammary infections (mastitis) and low concentrations during lactation. Interestingly, the pattern of mammary IGFBP-3 secretion mimics this pattern. Western ligand blotting with <sup>125</sup>I labeled Lf and transferrin (Tf) demonstrates that Lf, but not Tf specifically binds IGFBP-3. Reverse experiments utilizing <sup>125</sup>I labeled rhIGFBP-3 shows binding to Lf and not Tf. Lf competes in a dose dependent manner with IGF-I for binding to rhIGFBP-3 and can cause dose-related release of IGF from IGF:IGFBP-3 complexes. Furthermore, primary cultures of bovine mammary epithelial cells are stimulated to grow (10-30%) with concentrations of 0.5 to 6.5  $\mu$ M of holo-bLf even when a basal level of holo-Tf was applied to provide the cells with iron necessary for growth. Using fluorescent (FITC)-labeled holo-bLf and Texas Red rhIGFBP-3, we show that bovine primary epithelial cells translocate little of these proteins to the nucleus after 2 days of culture in serum free media (SFM). However, after 8 to 10 days of SFM culture and when the cells are synthesizing and secreting Lf into the media, as demonstrated by immunoblots, the cells are capable of translocating labeled bLf:IGFBP-3 complexes into the cell nucleus. This suggests that in addition to Lf and IGFBP-3, one or more components are necessary for nuclear translocation to occur. In summary, we have shown that Lf is an IGFBP-3 binding protein that competes with IGF. Additionally, the competitive displacement of IGFs from IGFBP-3 may account for the Lf enhancement of mammary cell growth. Finally, Lf appears to be a carrier for IGFBP-3, thereby facilitating its translocation to the nucleus of responsive mammary cells, offering the potential for additional mechanism(s) of regulation of mammary cell growth or apoptosis. From this, we hypothesize a new role for Lf in the regulation of the IGF system.

P2-585

**A NOVEL PROTEIN THAT INTERACTS SPECIFICALLY WITH INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 3.**

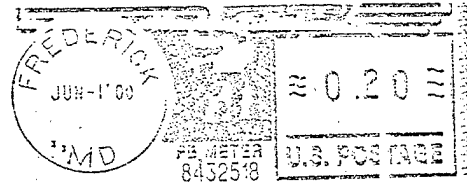
Angela R. Ingemann,\* Youngman Oh. Dept. of Pediatrics, Oregon Health Sciences University, Portland, OR

The human insulin-like growth factor binding protein (IGFBP) superfamily is comprised of 6 high affinity species (IGFBPs 1-6) and low affinity IGFBP-related proteins (IGFBP-rPs), of which there are nine to date. The IGFBPs are classically known to bind IGFs and modulate the IGF signalling system by controlling the availability of IGFs for the type I and type II IGF receptors. IGFBP-3, the predominant IGFBP in human serum, has recently been shown to exhibit IGF-independent biological activity. Data indicate that IGFBP-3 alone can inhibit the growth of various cancer cell lines, including breast and prostate. In particular, TGF- $\beta$ , retinoic acid-, and some antiestrogen-induced growth inhibition in breast cancer cells is mediated through IGFBP-3 action, at least in part. IGFBP-3 has also been shown to specifically bind the surface of these cells. Further, it has been demonstrated that IGFBP-3 induces apoptosis in prostate cancer cells. IGFBP-3, therefore, may play an important role in cell growth regulation.

This study reports the isolation and characterization of a novel protein which interacts specifically with IGFBP-3 in an IGF-independent manner. A mid-region fragment of IGFBP-3 was used as bait in the yeast two-hybrid system to identify interacting clones from an Hs578T human breast cancer cell cDNA library. Three individual cDNA clones demonstrated specificity for IGFBP-3 interaction when tested against lamin, a commonly used false-positive indicator. One of these, designated clone 4-33, represents a novel gene/protein. Northern analysis of clone 4-33 mRNA indicates wide distribution in human tissues. Expression was also detected in human breast and prostate cancer cell lines. Transiently expressed, GFP-fused clone 4-33 protein displays a strong and specific interaction with IGFBP-3 in a coimmunoprecipitation assay. Immunofluorescent detection of transiently expressed, hemagglutinin-tagged clone 4-33 protein reveals a perinuclear, and sometimes diffusely cytoplasmic localization pattern. This pattern may indicate distribution to the ER and subsequent trafficking to the cell membrane or secretion.

The wide distribution of clone 4-33 expression and specific interaction with IGFBP-3 present the possibility that this protein may play a ubiquitous role in the IGF-independent actions of IGFBP-3. We are currently expressing recombinant clone 4-33 protein for cellular studies and polyclonal antibody generation, to allow investigation of endogenous 4-33 protein function and its involvement in IGFBP-3 biological action.

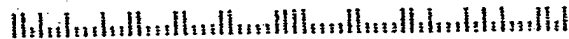
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**ACKNOWLEDGMENT OF RECEIPT OF PROPOSAL**

Your proposal to the Congressionally Directed Medical Research Program 2000 Prostate Cancer Research Program has been received. Your proposal identification number is:

PC001374

Preclinical Development of Antisense Therapeutics for Prostate Cancer Using Phosphorodiamidate Morpholino Oligomers

Please cite your proposal identification number in any further correspondence. You will not receive additional information until November 2000.

Thank you for your interest.

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